Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editors: Vinood B. Patel
Victor R. Preedy

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Vinood B. Patel · Victor R. Preedy
Editors

Biomarkers in Nutrition

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Biomarkers in Disease: Methods, Discoveries and Applications

Series Editors

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In the past decade there has been a major sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. New analytical methods that have been used to identify markers of disease and is suggested that there may be as many as 40 different platforms. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Nutrition

With 126 Figures and 109 Tables

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Preface

In recent years, there have been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies, as well as advances in chemistry and physics. This has led to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these progressions have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics, these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidence-based text that addresses these issues. Hence the book ***Biomarkers in Disease: Methods, Discoveries, and Applications: Biomarkers in Nutrition***. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and links with treatments. It links conventional approaches with new platforms.

In the present book, ***Biomarkers in Nutrition***, we have sections on:

1. ***Circulating and body fluid biomarkers***
2. ***Micronutrients and minerals***
3. ***Diets and macronutrients***
4. ***Genetic, molecular, and cellular variables***
5. ***Functional and physiological variables and platforms***
6. ***Biomarkers in specific conditions or scenarios***
7. ***Resources***

The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Editors recognize the difficulties in assigning chapters to parts of the book, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage and is well indexed.

The chapters are written by national and international experts. This book is designed for nutritionists, dietitians, clinical biochemists, health scientists, epidemiologists, researchers, doctors, and nurses, from students to practitioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and academic libraries as a reference guide.

London, UK
November 2022

Dr. Vinood B. Patel
Professor Victor R. Preedy

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About the Editors

Vinood B. Patel, BSc, PhD, FRSC, is currently Reader in Clinical Biochemistry at the University of Westminster. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for the diagnosis and prognosis of liver disease and understanding mitochondrial oxidative stress in Alzheimer's disease and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his PhD in protein metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized researcher and has several edited biomedical books related to the use or investigation of active agents or components as well as biomarkers. These books include *The Handbook of Nutrition, Diet, and Epigenetics; Biomarkers in Cancer; Biomarkers in Cardiovascular Disease;* and *Biomarkers in Liver Disease.* In 2014, Dr. Patel was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy, BSc, PhD, DSc, FRSB, FRSPH, FRCPath, FRSC, is Professor of Clinical Biochemistry and Pathology at King's College Hospital, Emeritus Professor of Nutritional Biochemistry at King's College London, and Visiting Professor at the University of Hull. Professor Preedy graduated in 1974 with an Honors Degree in Biology and Physiology with Pharmacology. He gained his University of London PhD in 1981. In 1992, he received his Membership of the Royal College of Pathologists and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then, he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public

Health and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the Cardiothoracic Institute, National Heart Hospital (part of Imperial College London), The School of Pharmacy (now Part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long-standing interest in biomarkers, especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 750 articles, which includes peer-reviewed manuscripts based on original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

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Part I

Circulating and Body Fluid Biomarkers

Advanced Glycation End Products as Biomarkers in Nutrition

1

Adina Bianca Boșca, Carmen Mihaela Mihu, and Aranka Ilea

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Abstract

The human body is exposed to dietary advanced glycation end products (AGEs) – dAGEs ingested with food and the endogenous AGEs, which are continuously formed in vivo. Clinical and experimental studies concluded that dAGEs, which are abundant in the Western diet, are directly absorbed in circulation, accumulate in the tissues, and add to the endogenous AGEs to significantly increase the systemic AGEs burden. The interaction between AGEs and the receptor – RAGE – is a key factor in the initiation of intracellular signaling pathways, with the subsequent formation of free radicals and the release of pro-inflammatory cytokines. The pro-inflammatory and prooxidant systemic effects of AGEs promote the development of chronic general diseases. Based on the correlations between AGEs intake, circulatory levels, and the risk of systemic pathology, AGEs measured in the body fluids could be used as biomarkers for the diagnosis and screening of metabolic syndrome and aging. The AGEs-restricted nutrition and physical exercise have beneficial effects and are recommended to promote health across all ages.

Keywords

Advanced glycation end products (AGEs) · Biomarkers · Low-grade chronic inflammation · Nutrition · Oxidative stress · Systemic pathologies

Abbreviations

AGEs	Advanced glycation end products
ALEs	Advanced lipoxidation end products
CML	Nε-Carboxymethyl-lysine
dAGEs	Dietary advanced glycation end products
eNOS	Endothelial NO synthase
hPEPT1	Peptide transporter1
hsCRP	High-sensitivity C-reactive protein
Lys	Lysine
MAPKs	Mitogen-activated protein kinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
PI3-K	Phosphatidylinositol 3-kinase
RAGE	Receptor advanced glycation end products
TLR4	Toll-like receptors 4
TNF-α	Tumor necrosis factor alpha

Introduction

Since their first discovery at the beginning of the twentieth century as compounds formed in foods during processing at high temperature, advanced glycation end products (AGEs) have gained more and more attention in the last decades, with the progress in knowledge regarding their sources, the physiopathological implications, and the role as biomarkers in aging and various systemic diseases.

Dietary AGEs (dAGEs) are products of the Maillard reaction (MR) formed by the thermally processing of food ingredients that represent major components of the Western nutrition.

Clinical studies demonstrated a statistical correlation between the tissue accumulation of AGEs and aging or various pathologies, but without a certain causal association with the disease. However, the association between dAGEs and multi-organ pathologies has been evidenced in experimental models (Cai et al. 2008, 2012).

Nutritional interventions limiting the dAGEs intake have been proved to be efficient in reducing the risk of obesity, diabetes, and diseases associated with the metabolic syndrome, as well as preventing premature aging.

AGEs: Definition and Historical Background

AGEs are a heterogeneous group of compounds formed by the nonenzymatic reaction of carbonyl group (C=O) in reducing sugars with free amino groups (NH_2-) of proteins, nucleic acids, or lipids. Formation of AGEs occurs through a series of reactions, leading to Schiff bases and Amadori products. The most representative AGEs are hydroimidazolone, Nε-carboxymethyl-lysine (CML), pentosidine, and glucosepane (Semba et al. 2010).

The reaction leading to AGEs formation was firstly described by Louis Camille Maillard in 1912, who observed that the browning of foods is the result of exposure of amino acids and sugars such as glucose to high temperatures (Maillard 1912). This process, later known as Maillard reaction, was initially studied as being responsible for the characteristic color, flavor, and taste of foods (Cho et al. 2007), but was later described as occurring in vivo, and termed glycation (Ulrich and Cerami 2001). Initially, it was considered that glycation of macromolecules and binding of AGEs to specific receptors on the cell surface (RAGE) were mechanisms for clearance and degradation of AGEs by macrophages. Instead, the AGEs-RAGE interaction induces the post receptor signaling with subsequent activation of an intracellular pathogenetic cascade leading to cellular dysfunction (Bierhaus et al. 2005).

In the early 1990s, the implication of dAGEs in systemic pathologies was omitted because of the presumed low absorption; however, recent epidemiological studies demonstrated the risk of cardiovascular disease, liver and kidney diseases, neurodegenerative diseases, diabetes complications, and bone and muscle diseases in elders with high levels of circulating CML, one of the major AGEs (Semba et al. 2010; Delgado-Andrade 2016).

AGEs Sources and Formation

AGEs accumulating in the human body derive from two sources: the exogenous dAGEs are found in foods and are ingested, whereas endogenous AGEs are formed by a series of reactions in the living tissues (Semba et al. 2010).

Exogenous dAGEs are formed in the food by the MR or nonenzymatic browning that occurs during preparation of sugars and proteins at high temperatures and is responsible for the characteristic color and flavor of the thermally processed foods (Henle 2005). MR consists of the following steps: (i) the formation of an Amadori compound by the reaction between the carbonyl group of a reducing sugar (e.g., glucose, lactose, maltose) and an amino group from an amino acid, peptide, or protein, followed by the rearrangement; (ii) Amadori products undergo degradation by heating or longer storage and form 1,2-dicarbonyls, such as glyoxal and methyl-glyoxal (MG); (iii) dicarbonyls will form low-molecular heterocyclic flavor compounds and are also highly reactive with side chains lysine and arginine of proteins and will form peptide-bound amino acid derivates as end products of the MR (Henle 2005) (Fig. 1).

Common industrial food processing that involves dehydration, ionization, or irradiation and home cooking at high temperature, roasting or frying, significantly increase the amount of dAGEs (Luft et al. 2016). Additionally, cigarettes are an important source of AGEs due to thermal processing of the tobacco and the combustion during smoking (Băbțan et al. 2019a). Cerami et al. (1997) demonstrated that glycotoxins from cigarette smoke inhaled into the lungs participate in the formation of AGEs and affect the alveolar cells and can also pass into the blood.

The variety of food ingredients abundant in fat and sugar and the processing conditions explain the large content of AGEs in the Western diet (Delgado-Andrade 2016). The study conducted by Hull et al. (2012) on food categories demonstrated the highest content of CML in dairy, bakery products, sweets, and snacks processed at high temperature, compared with low amounts in meat, fish, fruits, and vegetables cooked at moderate temperatures by boiling, steaming, or pasteurization (Hull et al. 2012; Uribarri et al. 2010) (Table 1).

In biological systems, Maillard-like reaction, also known as glycation, results in the formation of endogenous compounds: AGEs and advanced lipoxidation end products (ALEs). The in vivo formation of AGEs/ALEs compounds is promoted by obesity and the related metabolic syndrome, characterized by hyperglycemia, hyperlipidemia, and oxidative stress (Delgado-Andrade 2016).

The in vivo MR resulting in endogenous AGEs involves three stages: (i) the formation of a Schiff base by the nonenzymatic reaction between glucose and the amino acid (usually lysine or arginine) in a protein, lipid, or nucleic acid (this phase occurs within hours to days and is reversible); (ii) the rearrangement of Schiff bases with the formation of Amadori products, or early glycation products, which are more stable, e.g., hemoglobin A1c (this reaction occurs within days or weeks, but it is still reversible); and (iii) accumulated Amadori products that undergo complex rearrangement by oxidation, hydration, or reduction and will result in crosslinked compounds, the AGEs which are very stable (these reactions occur in weeks or months and are irreversible) (Luevano-Contreras and Chapman-Novakofski 2010).

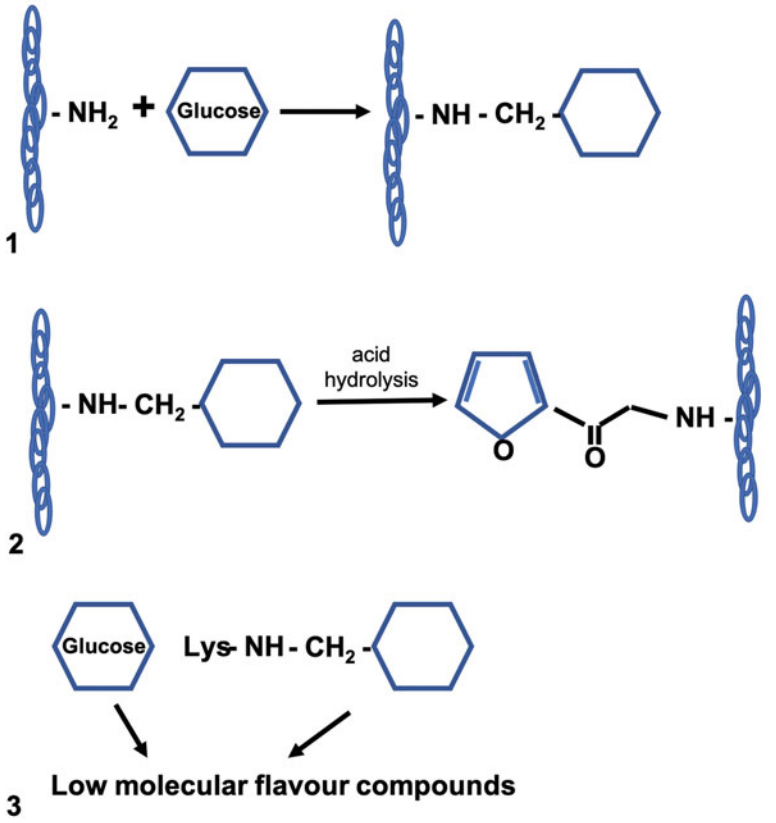

Fig. 1 **The formation of the dAGEs.** 1, The first stage is the reaction between glucose and the amino group of a peptide, followed by rearrangement. 2, The degradation of the Amadori products to 1,2-dicarbonyls. 3, Heterocyclic flavor compounds will react with amino acids to form peptide-bound amino acid derivates. Lys, lysine

Table 1 The dietary AGEs content in common food categories in the Western diet

Food category	mg CML/100 g food Mean ± SD
Meat and fish	0.86 ± 1.08
Meat dishes	2.42 ± 9.16
Dairy products	0.44 ± 0.45
Breads and savory biscuits	1.29 ± 1.22
Cereals	2.55 ± 1.36
Potatoes rice and pasta	0.13 ± 0.12
Coffee	0.01 ± 0
Fruits and vegetables	0.13 ± 0.03
Sweets and snacks	1.81 ± 1.73
Soups and sauces	0.39 ± 0.38

Mean ± SD (standard deviation) of the CML, Nε-carboxymethyl-lysine content of foods, expressed per 100 g food (Hull et al. 2012)

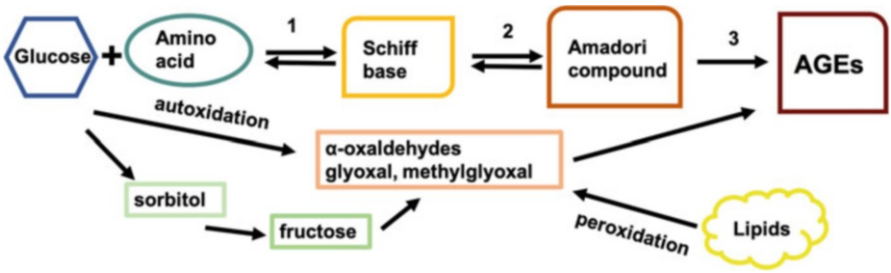

Fig. 2 The formation of the endogenous AGEs. 1, In the first stage, the nonenzymatic reaction between glucose and an amino acid will form a Schiff base; this reaction occurs in hours or days and it is reversible. 2, In the second stage, the rearrangement of Schiff bases results in Amadori products; this stage occurs in days to weeks and it is reversible. 3, In the third stage, Amadori products undergo complex transformations and will irreversibly form AGEs in weeks or months. By autoxidation of glucose and peroxidation of lipids, the α-oxaldehydes are formed. The polyol pathway involves transformation of glucose into sorbitol and fructose and then results in α-oxaldehydes, which will further become AGEs

Besides MR, other pathways leading to endogenous AGEs formation are the autoxidation of glucose and the peroxidation of lipids and the polyol pathway. Glucose autoxidation and lipid peroxidation occur due to oxidative stress leading to the formation of dicarbonyl derivatives – α-oxaldehydes such as glyoxal or methylglyoxal (Uribarri and Tuttle 2006). The polyol pathway involves the aldose reductase that converts glucose into sorbitol which is transformed into fructose by the sorbitol dehydrogenase. Fructose metabolites are converted into α-oxaldehydes and interact with monoacids to form AGEs (Lorenzi 2007) (Fig. 2).

AGEs derived from the two sources sum up to form the total pool of circulating AGEs. The exogenous serum AGEs are represented by the dAGEs which are absorbed as such and the AGEs formed in vivo based on the absorbed dicarbonyl compounds. The endogenous circulating AGEs can form based on plasma proteins or peptides derived by proteolysis from the extracellular and intracellular proteins (Uribarri et al. 2005; Degen et al. 2012).

A recent hypothesis suggests that the AGEs from the two different sources have a synergistic activity, leading to deleterious effects due to systemic glycoxidant burden and oxidative stress (Somoza 2005; Cai et al. 2004).

Bioactivity of AGEs and Impact of dAGEs on Health

In the late1970s, the studies on the formation of AGEs in living tissues started to clarify the mechanisms implicated in diabetes and aging (Semba et al. 2010; Bunn et al. 1978; Ulrich and Cerami 2001).

Alteration in structure and function is the result of the progressive and irreversible deposition of AGEs in tissues and organs (Singh et al. 2001), since AGEs exert significant prooxidant and pro-inflammatory effects (Negrean et al. 2007).

Accumulation in Tissues and Organs

The bioavailability of dAGEs depends on the solubility after digestion (Delgado-Andrade et al. 2009) and on the molecular weight and the free or protein-bound form (Faist and Erbersdobler 2001). The early MR products, such as Amadori compounds, can be partially reverted by the stomach acid, but CML, an advanced compound, is more stable (Delgado-Andrade 2016). Moreover, low-molecular-weight free AGEs are easily absorbed and have a faster metabolic transit and elimination compared with protein-bound forms (Poulsen et al. 2013). Clinical and experimental studies reported different rates of absorption, biodistribution, and excretion of CML through urine and feces, depending on the intake amounts, free or protein-bound forms (Delgado-Andrade 2016; Alamir et al. 2013). The incomplete elimination of ingested dAGEs is due to multiple factors, including the digestion of the compounds in the gastrointestinal tract, the biotransformation in the internal medium after absorption, and the limited capacity of the body to detoxify; these factors are responsible for the accumulation of AGEs, with the subsequent impact on tissue physiology and morphology (Roncero-Ramos et al. 2013; Förster et al. 2005).

Mechanisms Underlying the Pathogenetic Pathways of AGEs

The molecular mechanisms of glycation-induced pathogenesis are not completely understood. However, several pathogenetic pathways have been demonstrated: (i) protein glycation leads to the formation of toxic AGEs that accumulate in various tissues and organs; (ii) AGEs alter the structure or conformation of tissue proteins, resulting in impaired function; (iii) AGEs induce tissue modifications by inter- and intramolecular crosslinking; (iv) the interaction of AGEs with the specific RAGE receptors on the cell surface initiates the activation of an intracellular cascade leading to increased production of free radicals – reactive oxygen species (ROS) and pro-inflammatory mediators (Wu et al. 2011).

The pathogenetic pathways of AGEs involve two mechanisms (Luevano-Contreras and Chapman-Novakofski 2010): one of the mechanisms is independent of receptors and consists of the accumulation of AGEs in the extracellular matrix with the subsequent damage of tissue proteins; the other mechanism is dependent on the interaction between the AGEs and receptors and ligand proteins, such as RAGE or Toll-like receptors 4 (TLR4). The interaction between AGEs and RAGE, the specific receptors, induces subsequent activation of the nuclear factor-kappa B (NF-κB) pathway through the mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-kinase (PI3-K). The nuclear translocation of the activated NF-κB further induces the transcription of genes for the synthesis of pro-inflammatory cytokines, growth factors, and adhesive molecules, with prolonged cellular dysfunction and localized tissue destruction (Bierhaus et al. 2005; Lukic et al. 2008). Furthermore, NF-κB promotes RAGE expression by positive feedback and maintains the pro-inflammatory state. The interaction between AGEs

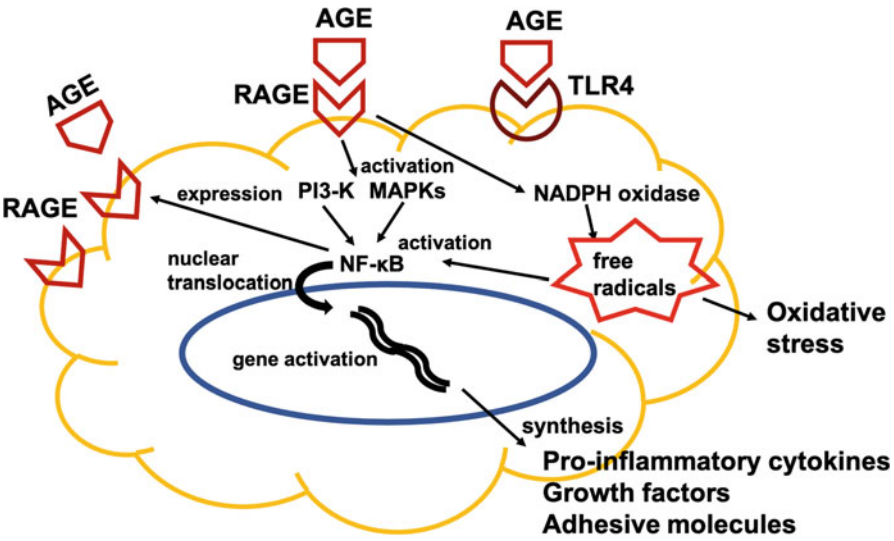

Fig. 3 The pathogenetic mechanism based on the interaction between AGEs and RAGE. AGEs may bind to TLR4. Upon binding to the specific receptors, AGEs trigger an intracellular pathogenetic cascade that involves the activation and the nuclear translocation of the transcription factor NF-κB through MAPKs and PI3-K. NF-κB further promotes the activation of the genes for pro-inflammatory cytokines, growth factors, and adhesion molecules and additionally promotes RAGE expression. The AGEs binding to RAGE also activate the NADPH oxidase with the consequent production of superoxide, leading to oxidative stress. Intracellular free radicals further activate NF-κB. AGEs advanced glycation end products, RAGE receptors for AGEs, TLR4 toll-like receptor 4, NF-κB nuclear factor-kappa B, MAPKs mitogen-activated protein kinases, PI3-K phosphatidylinositol 3-kinase, NADPH oxidase nicotinamide adenine dinucleotide phosphate oxidase

and RAGE also increases the production of superoxide by activating the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and induces oxidative stress. The intracellular free radicals are responsible for activation of NF-κB (Basta et al. 2005; Wautier et al. 2001; Lin et al. 2009) (Fig. 3).

Biological Effects of dAGEs

The body's AGEs pool is the sum of the dAGEs absorbed in circulation and the endogenous AGEs produced in vivo (Fig. 4).

Several studies investigated the gastrointestinal absorption, metabolism, and urinary or fecal excretion of dAGEs. Koschinsky et al. (1997) estimated that about 10% of the ingested AGEs were absorbed into circulation, of which two-thirds are deposited in the tissues and the rest of one-third is eliminated. Even though the intestinal absorption of dAGEs is low, there is a significant increase in plasma AGEs levels within 2 h after the ingestion of a single AGEs-rich meal (Koschinsky et al. 1997). Uribarri et al. (2005) demonstrated the correlation between dAGEs intake and

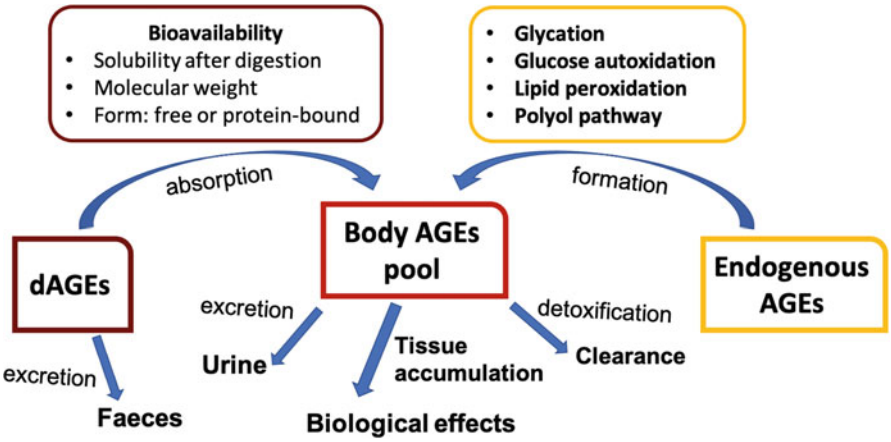

Fig. 4 Factors contributing to the body's AGEs pool: the dAGEs, ingested with food, and the endogenous AGEs produced in vivo. The dAGEs are partially absorbed in the circulation, depending on their bioavailability, and most are excreted. Endogenous AGEs are formed by glycation, glucose autoxidation, lipid peroxidation, and the polyol pathway. The circulating AGEs can be partially eliminated by renal excretion or by enzymatic clearance, and the rest accumulate in the tissues and exert multiple biological effects. dAGEs dietary advanced glycation end products

the circulating levels of these compounds, but also the association with inflammatory markers, in healthy individuals.

However, it is difficult to establish the bioavailability of dAGEs, since they are very heterogeneous, and only three compounds have been well characterized and studied: CML, pyrraline, and pentosidine. Moreover, clinical data reported variable absorption rates of dAGEs. The study conducted by Geissler et al. (2010) demonstrated that intestinal absorption of pyrraline was mediated by the peptide transporter hPEPT1.

The renal excretion is an important defense mechanism preventing the accumulation of AGEs, under normal conditions. The imbalance caused by high endogenous AGEs production and/or exogenous intake and an insufficient renal clearance leads to accumulation of AGEs and the subsequent pathological changes (Vlassara and Striker 2007; Peppa et al. 2008).

The detoxification of AGEs is performed by several enzymes, glyoxalases I and II and carbonyl reductase, and prooxidant and pro-inflammatory effects of glycation are counteracted by the anti-inflammatory AGER1 receptor (Vlassara et al. 2009; Vlassara and Striker 2007).

Firstly, consumption of ultra-processed foods that are rich in dAGEs is associated with weight gain due to the excessive energy intake (Sohouli et al. 2020).

Secondly, after ingestion, dAGEs are directly absorbed in circulation and exert prooxidant and pro-inflammatory effects (Uribarri et al. 2007a) leading to oxidative stress and low-grade chronic inflammation. The activation of signaling cascade RAGE/TLR4-NF-κB-ROS (Ott et al. 2014) plays important roles in pathogenesis of obesity and related diseases (Ajith and Vinodkumar 2016).

Several studies demonstrated that dAGEs have an effect on the insulin-sensitive tissues and induce insulin resistance, dyslipidemia, and atherosclerosis demonstrated by the variation of the hormones associated with the activity of adipose tissue: adiponectin and leptin (Yadav et al. 2013). Increased insulin levels and insulin resistance lead to reduced lipolysis and increased lipogenesis in adipocytes, explaining the weight gain (Kolb et al. 2018).

Hypothalamic inflammation is caused by high intake of dAGEs and increased accumulation of CML in neurons, leading to impaired control of energy metabolism (Gao et al. 2017).

The cumulative effects of these mechanisms, combined with genetic predisposition and environmental factors, contribute to the pathogenesis of multisystem dysfunction and diseases.

Implication of dAGEs in Aging and Pathological Conditions

Premature Aging and Age-Related Diseases

The physiological aging is accompanied by intracellular and extracellular accumulation of CML and other AGEs (Băbțan et al. 2019b) that exert deleterious effects leading to structural and functional impairment and higher risk of developing age-related chronic diseases (Semba et al. 2010) (Fig. 5).

The aspect and rigidity of aged skin is the consequence of nonenzymatic glycation of collagen in the dermis and formation of intermolecular crosslinks that alter the normal turnover (Schleicher et al. 1997).

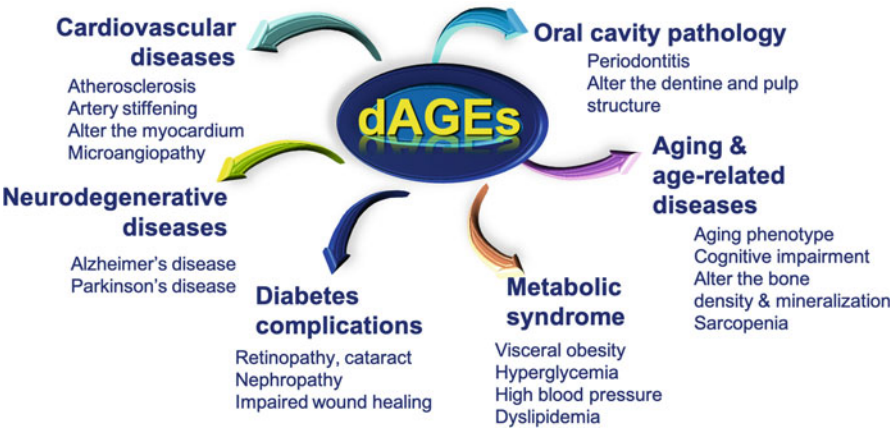

Fig. 5 Implication of dAGEs in systemic diseases and aging. dAGEs dietary advanced glycation end products

Cardiovascular changes in elders, including vascular stiffening, endothelial dysfunction, and abnormal diastolic relaxation, were associated with progressive accumulation of AGEs (Zieman and Kass 2004).

Sarcopenia in older populations is multifactorial, caused by hormonal changes, high levels of oxidative stress and inflammation, decrease in blood supply, and inactivity (Perrini et al. 2010; Sakuma and Yamaguchi 2010). Several studies reported the possible contribution of AGEs to the age-related decline in muscle strength (Haus et al. 2007; Dalal et al. 2009).

Aging, as well as the accumulation of AGEs, could be a risk factor for the cognitive decline and Alzheimer's disease in elderly people, due to the prooxidant and the pro-inflammatory effects (Cruz-Sanchez et al. 2010; Candela et al. 2010). Moreover, a link between diabetes and Alzheimer's disease has been hypothesized, since some of the biological mechanisms are similar: impaired glucose metabolism and insulin signaling leading to accumulation of AGEs, oxidative stress, and the pro-inflammatory state (Sims-Robinson et al. 2010).

In older adults, the imbalance between AGEs intake and clearance, caused by a decrease in renal function, can lead to increased levels of glycotoxin, and markers of inflammation and oxidative stress, as demonstrated by Uribarri et al. (2007b).

Metabolic Syndrome

The metabolic syndrome encompasses multiple interrelated cardio-metabolic risk factors: abdominal obesity, hyperglycemia, high blood pressure, and dyslipidemia (Lusis et al. 2008). These risk factors are implicated in the development of chronic systemic diseases, such as cardiovascular diseases, type 2 diabetes mellitus, and obesity (Watanabe and Kotani 2020). Pathogenesis of metabolic syndrome is multifactorial and involves environmental factors, including diet, physical activity, smoking, as well as genetic predisposition (Alberti et al. 2009; Ottum and Mistry 2015; Galbete et al. 2013). Both dAGEs and endogenous AGEs have noticeable pro-inflammatory and prooxidant effects leading to low-grade chronic systemic inflammation and oxidative stress, the main pathogenetic mechanisms underlying the diseases associated with the metabolic syndrome (Uribarri et al. 2015).

Diabetes and Diabetic Complications

The interrelation between AGEs and diabetes has been well defined, since the indicator of glycemia in diabetic patients is hemoglobin A1c, an early glycation product (Henle 2005). In diabetic patients, the high glycemic levels promote increased glycation, mainly in the insulin-independent tissues, including erythrocytes, vascular endothelium, peripheral nerves, lenses, and kidneys. The macrovascular and microvascular pathology were associated with high glucose levels, and the intracellular accumulation of glucose promotes increased protein glycation (Luevano-Contreras and Chapman-Novakofski 2010). Moreover, the glycation of

the proteolytic enzymes responsible for protein turnover results in inefficient removal of altered proteins and accumulation of larger amounts of AGEs (Morgan et al. 2002). Experimental studies demonstrated that high dAGEs intake increased serum CML levels, aggravated diabetes, and accelerated the progression of associated complications, including delayed wound healing, nephropathy, and retinopathy (Peppa et al. 2009; Hofmann et al. 2002; Zheng et al. 2002; Vlassara and Striker 2013).

Cardiovascular Diseases

The collagen glycation in the wall of the blood vessels causes arterial stiffening, atherosclerosis, and microangiopathy (Aso et al. 2000; Semba et al. 2009a), and intracellular accumulation of AGEs alters the function of the myocardium. AGEs induce endothelial activation and modify the low-density lipoproteins (LDL), which become glycated and crosslink with the collagen in the arterial wall, with the subsequent formation of the atherosclerotic plaque (Bucala et al. 1994; Zieman and Kass 2004). These changes occur in diabetic patients but can also be associated with high dAGEs intake (Luevano-Contreras and Chapman-Novakofski 2010). Other mechanisms involved in the pathogenesis of cardiovascular diseases are the decrease in nitric oxide synthesis in the endothelium due to reduced half-life of endothelial NO synthase (eNOS) leading to impaired vasodilatation (Xu et al. 2003) and the chelation of the redox-active iron and copper ions, which are bound by heavily glycosylated collagen and elastin within the arterial wall (Qian and Eaton 2000). In postmenopausal women, the risk of cardiovascular events caused by the increase in testosterone and free androgen levels was positively associated with higher levels of circulating AGEs (Diamanti-Kandarakis et al. 2010).

Neurodegenerative Diseases

Parkinson's, Huntington's, and Alzheimer's diseases are characterized by the abnormal protein accumulation or aggregation. Since the brain tissue has low antioxidant capacity, it is vulnerable to oxidative damage (Schulz et al. 2000). The local oxidative stress induces the formation of endogenous AGEs that further promote AGEs production by a positive feedback mechanism (Vicente Miranda and Outeiro 2010). The neurodegenerative processes could be the result of AGEs-mediated neurotoxicity, because the increased amounts of AGEs promote the formation of protein deposits due to the crosslinking between proteins and peptides that are resistant to proteases (Li et al. 2012a). In Alzheimer's disease, increased levels of CML in neurons (Moreira et al. 2005) suggest that AGEs participate in the hyperphosphorylation of the tau protein in the neurofibrillary tangles (Li et al. 2012b) and can also act as chelators of the redox-active metal ions in the plaques (Smith et al. 1997). Moreover, Cai et al. (2014) demonstrated the role of dAGEs in pathogenesis of Alzheimer's disease. They experimentally induced a dementia-like syndrome by a

diet rich in methyl-glyoxal, a nutrient-bound AGE, and observed increased deposits of AGEs and amyloid-β42 in the brain, associated with gliosis, and cognitive deficit. RAGE is implicated in the transport of amyloid peptides across the blood-brain barrier (Candela et al. 2010). In patients with Parkinson's disease, increased AGEs levels and RAGE expression in the frontal cortex promote the abnormal accumulation of modified proteins (Li et al., 2012a).

Renal Disease

The increased levels of AGEs could explain the association between type 2 diabetes and renal disease. Semba et al. (2009b) reported high levels of circulating CML in patients with chronic kidney disease. The clinical study performed by Vlassara et al. (2009) reported similar results and demonstrated that serum levels of CML and MG were negatively correlated with the glomerular filtration rate.

Oral Diseases

Oral tissues are exposed to both exogenous and endogenous AGEs that accumulate in the dental tissues: dentine and pulp, as well as in the periodontium. Crosslinking between AGEs and collagen fibers in the intertubular dentine and in the connective tissue of the dental pulp alters the morphology and mechanical properties. The deposition of high amounts of AGEs in the dentine was related to the increased hardness and dark discoloration that occur with age (Ilea et al. 2018; Miura et al. 2014). Additionally, AGEs accumulated in the dentine extract the Ca^{2+} ions from the calcified matrix and promote their precipitation in the dentinal tubules, further increasing the hardness of dentine and the risk of fracture. The yellow to brown dentine discoloration associated with the presence of AGEs is time and dose dependent. Crosslinking of collagen in the periodontal ligament alters the attachment to the tooth surface and aggravates periodontitis, with increased risk of tooth loss. In an animal model, the expression of CML in dental and periodontal tissues was shown to be increased with age (Băbțan et al. 2019c). Moreover, AGEs cause an imbalance in bone metabolism, by activating the osteoclasts and inhibiting the osteoblasts, thus promoting the resorption of the alveolar bone (Ilea et al. 2018). Saliva contains mucins, proteins that can be glycosylated, and the levels of salivary AGEs have been correlated with the serum levels (Ilea et al. 2018; Garay-Sevilla et al. 2005).

Strategies to Reduce AGEs Load

In order to reduce AGEs accumulation, several approaches have been proposed: the nutritional intervention to limit the dAGEs intake, the physical exercise, and some pharmacological agents.

The Effects of Nutritional Intervention on Health Outcomes

Clinical and experimental interventional studies demonstrated that lower intake of dAGEs reduced circulating and urinary AGEs, improved insulin resistance by influencing the adiponectin and leptin levels, and reduced the inflammatory and oxidative stress markers in overweight patients (Luevano-Contreras et al. 2012; Uribarri et al. 2011).

The low AGEs diet has impact on the hormones implicated in the metabolism of adipose tissue by lowering the synthesis of leptin and increasing the adiponectin levels, compared with the high AGEs diet (Sohouli et al. 2020).

The meta-analysis conducted by Sohouli et al. (2020) compared the effect of low and high AGEs diet on obesity, taking into consideration the variation of anthropometric parameters. Low AGEs intake induced a significant decrease body mass index (BMI), which was more pronounced in overweight and obese patients (Sohouli et al. 2020); the weight was significantly reduced, regardless of the initial overweight or obese status. The waist circumference (WC) was not significantly influenced by the amount of AGEs intake.

Clinical studies in patients with uremia and diabetes indicated that a low dAGEs intake resulted in reduction in AGEs levels and inflammatory markers such as tumor necrosis factor alpha (TNF-α) and high-sensitivity C-reactive protein (hsCRP) (Uribarri et al. 2003).

Physical Exercise

In an experimental study, exercise-trained rats showed improved cardiac performance due to decreased pathological cross-linking in the myocardium, more efficient cardiac contractility (Choi et al. 2009). Boor et al. (2009) compared exercise-trained rats with sedentary rats and reported decreased circulating levels of CML and lower renal accumulation.

Studies on human subjects demonstrated a decrease in AGEs and malondiadehyde in healthy individuals older than 45 years, after 12 months of tai chi practice (Goon et al. 2009). In a group of heathy middle-aged women, the serum AGEs levels were negatively correlated with the daily step count (Yoshikawa et al. 2009).

Pharmacological Agents

Saha et al. (2010) reported decreased urinary levels of CML after administration of candesartan, an inhibitor of angiotensin receptor for 12 weeks, in patients with diabetes complicated with diabetic nephropathy. Metformin was reported to reduce the serum AGEs levels in diabetic patients (Isoda et al. 2006) and in women with polycystic ovary syndrome (Diamanti-Kandarakis et al. 2007a). Orlistat, a lipase inhibitor, reduced the absorption of dAGEs and lowered the serum AGEs levels (Diamanti-Kandarakis et al. 2007b).

Corman et al. (1998) demonstrated, in a long-term clinical study, that the administration of aminoguanidine, a scavenger of α-dicarbonyl, inhibited the AGEs formation and prevented the age-related myocardium hypertrophy and vascular stiffness.

The Role of AGEs as Biomarkers in Nutrition

The circulating levels of AGEs are dependent on several factors: the exogenous intake, the endogenous production, the renal and fecal excretion, and the enzymatic clearance.

Extensive data indicate that the AGEs levels in serum/plasma, urine, and saliva vary with the dietary intake and are correlated with the age and the health status reflected by the markers of inflammation and oxidative stress.

Moreover, clinical and experimental studies demonstrated that an AGEs-rich diet was associated with increased serum levels and tissue accumulation of CML, one of the major AGEs; thus, the serum-free CML could be indicative for the dAGEs intake (Uribarri et al. 2010; Somoza et al. 2006; Alamir et al. 2013).

Circulating levels of AGEs and specifically CML, even though increased in older people, are correlated with the markers of inflammation and oxidative stress across all ages. Moreover, circulating levels of AGEs and markers of inflammation and oxidative stress are directly influenced by the dAGEs and independent of age and the calorie intake.

The positive association between dAGEs ingestion, serum AGEs levels, visceral fat, cardiovascular diseases, and insulin resistance suggests the implication of these compounds in metabolic syndrome and the role as biomarkers of systemic pathologies.

Applications to Prognosis

Controlling/Reducing the Amount of dAGEs Intake Could Help to Improve Health

Changes in the quality of nutrition by reducing the dAGEs intake could have major impact on health and longevity. This purpose can be easily achieved by modifying food processing conditions in order to prevent the formation of AGEs (Uribarri et al. 2010). Consumers should be educated on how to use different cooking methods that involve lower temperature, high moisture, and shorter time (Hull et al. 2012). It was demonstrated that cooking the same food under low temperature and high moisture conditions, by boiling or poaching, the final product contained lower levels of CML compared with frying or baking at high and dry temperature (Delgado-Andrade et al. 2009).

Moreover, it is important to estimate the potential amount of certain toxic compounds found in various food products. A specific dAGE, methylglyoxal, has been indicated to promote oxidative stress and low-intensity chronic

inflammation and is considered as a modifiable risk factor for prevention of neurodegenerative diseases and the metabolic syndrome (Cai et al. 2014). CML is the most investigated compound in foods and has been reported to be present in a wide variety of foods, including powdered infant formula, bakery products, cereals, raw and processed milk, and grilled or fried meat and fish (Hull et al. 2012).

Therefore, in order to prevent the nutrition-associated diseases, it is important to know the potential dAGEs intake from the consumed food.

On the other hand, association of inhibitors that react with active dicarbonyls and prevent the formation of AGEs could also be beneficial. These are nucleophilic reagents including pyridoxamine (Carvalho et al. 2001) or aminoguanidine (Price et al. 2001). It was demonstrated that natural antioxidants, such as flavonoids and polyphenols, scavenge the free radicals and thus limit the formation of CML (Uribarri et al. 2010).

By normalizing the levels of obesity-related hormones, AGE-restricted diet could prevent diabetes and the associated complications, such as macrovascular and microvascular changes, diabetic retinopathy and nephropathy, peripheral neuropathy, and impaired wound healing (Hofmann et al. 2002; Zheng et al. 2002).

Lifestyle modification with the practice of short- and long-term moderate physical exercise demonstrated to be beneficial in reducing the levels of circulating AGEs.

Thus, reducing the AGEs pool by dAGEs restriction and physical exercise could be a safe economic policy to prevent age-related diseases, as well as metabolic syndrome and the associated complications across all ages.

Applications to Other Diseases or Conditions

Diagnostic Approaches Based on Measurement of AGEs Levels in the Biofluids

Measurement of AGEs levels in biofluids could be useful for (i) determining the dAGEs intake and the endogenous production, (ii) assessing the aging process and the onset of age-related diseases, and (iii) evaluating the health status and risk of developing chronic pathologies: metabolic syndrome and the associated complications (Băbțan et al. 2019c; Ilea et al. 2018).

Saliva is a biofluid with a complex composition that mirrors the health status and contains various components, including AGEs (Ilea et al. 2019). Moreover, it can be easily and noninvasively sampled for the assessment of various biomarkers. By establishing a correlation between salivary and serum AGEs levels, saliva could be a valuable tool for the diagnostic or screening of systemic pathologies. Furthermore, the development of biosensors for the detection of salivary AGEs as prognostic and/or diagnostic biomarkers in nutrition opens new perspectives in monitoring normal and pathological conditions.

Mini-Dictionary of Terms

- ***Advanced glycation end products (AGEs)*** Final products of glycation, a reaction between a carbohydrate and an amino acid or a protein.
- ***Maillard reaction.*** The process that occurs during food processing at high dry temperature. It is responsible for the brown color and the characteristic flavor of bakery products or roasted meat.
- ***Glycation.*** The Maillard-like reaction that occurs in the biological systems.
- ***Nε-Carboxymethyl-lysine.*** One of the major AGEs that is found in processed foods and is also formed in the body.
- ***Biomarker.*** A molecule found in biofluids and/or in tissues that can indicate a normal condition or a pathological change. Measuring the levels of biomarkers is useful for the diagnosis or monitoring of a disease or as a screening tool.
- ***Metabolic syndrome.*** A group of conditions or factors that include abdominal obesity, hyperglycemia, high blood pressure, and dyslipidemia. These factors are associated with increased risk of developing chronic systemic diseases such as cardiovascular disease, diabetes complications, and type 2 diabetes.

Key Facts of Advanced Glycation End Products (AGEs)

- *Human body is exposed to two sources of advanced glycation end products (AGEs).*
- *The exogenous sources are the dietary AGEs – dAGEs, compounds formed in the thermally processed foods.*
- *Endogenous AGEs are continuously produced in the human body.*
- *The dAGEs are abundant in the Western nutrition, due to the variety of food ingredients and the processing conditions.*
- *Both dAGEs and endogenous AGEs progressively accumulate in the body and have a negative effect on health.*

Key Facts of Impact of Dietary Advanced Glycation End Products (dAGEs) on Health

- *After ingestion, dAGEs are partially eliminated, but about 10% are absorbed in the gastrointestinal tract and enter into the systemic circulation.*
- *At tissue level, AGEs exert pro-inflammatory and prooxidant effects. These pathogenic mechanisms play an important role in the aging process and the chronic diseases.*
- *The high dAGEs intake and the accumulation of AGEs are risk factors for premature aging and general diseases, such as cardiovascular diseases, Parkinson's disease or Alzheimer's disease, obesity, and diabetes complications.*

- *Based on the correlation of dAGEs intake with the levels in the body fluids, these compounds could be used as biomarkers in nutrition.*
- *The AGE-restricted nutrition and physical exercise have beneficial health effects.*

Summary Points

- *Humans are exposed to two sources of AGEs: the dietary AGEs – dAGEs – found in food and the endogenous AGEs, formed in the body.*
- *The Western diet is rich in dAGEs, due to the high variety of food ingredients and the processing at high temperature.*
- *After absorption in circulation, dAGEs accumulate in the tissues and increase the systemic AGEs burden.*
- *The interaction between AGEs and the receptor – RAGE – results in pro-inflammatory and prooxidant effects; thus, AGEs are involved in the low-intensity chronic inflammation and the oxidative stress.*
- *The pathogenetic pathways initiated by dAGEs underlie aging and various multi-system pathologies, such as the metabolic syndrome, type 2 diabetes and diabetes complications, cardiovascular diseases, and neurodegenerative diseases.*
- *The dAGEs intake is correlated with the circulating AGEs levels and the risk of chronic diseases.*
- *Strategies to reduce the AGEs load in the body include nutritional interventions to reduce the amount of dAGEs, practicing physical exercise, and administration of pharmacological agents.*
- *The levels of AGEs in the body fluids could be used as biomarkers in systemic pathologies and aging.*
- *Monitoring AGEs salivary levels could help to improve nutrition in order to promote health.*

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Trimethylamine N-Oxide (TMAO) as a Biomarker

2

Features and Applications [to nutrition]

Rosita Gabbianelli and Laura Bordoni

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Abstract

The interaction between diet, microbiome, and noncommunicable disease onset is gaining growing attention. The trimethylamine N-oxide (TMAO) is a gut microbiota derivative that has been suggested as a potential regulator of human health, especially (but not exclusively) for its association with cardiovascular diseases. It derives from the trimethylamine (TMA), which is produced by the gut microbiome from dietary precursors, such as choline, betaine, and L-carnitine. Due to the potentially harmful effects of TMAO, strategies aimed to reduce circulating TMAO levels (ranging from dietary restrictions or supplementation to pharmacological treatments) have been proposed. Moreover, TMAO has been suggested as a biomarker of disease onset and prognosis. Nevertheless, contrasting evidence can be found in the literature, and mechanistic explanations or causal demonstrations of the association between the TMA/TMAO metabolism and diseases are still missing. Thus, despite promising findings, the history of TMAO might be more complex than initially hypothesized, and further studies are necessary to promote their translation into clinical practice.

Keywords

Trimethylamine N-oxide · Gut microbiota · Molecular nutrition · Cardiovascular disease · Noncommunicable diseases · Nutrigenomics · Diet · Choline · Carnitine · Betaine · Atherosclerosis · Nutraceuticals · Probiotics

Abbreviations

CKD	Chronic kidney diseases
CVD	Cardiovascular disease
FMO	Flavin monooxygenases
MetS	Metabolic syndrome
NAFLD	Nonalcoholic fatty liver disease
NCD	Noncommunicable disease
STEMI	ST-segment elevation myocardial infarction
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide

Introduction

Both the diet and the gut microbiota play a role in modulating the predisposition to noncommunicable diseases (NCDs), which represent a major burden for the modern society (Beaglehole et al. 2011). Among the numerous dietary molecules that are metabolized by the gut microbiome before entering into the blood circulation, there is trimethylamine (TMA). TMA is absorbed and then oxidized into trimethylamine N-oxide (TMAO) in the liver. Since circulating TMAO levels have been associated with several NCDs, TMAO has recently grabbed the attention of the scientific

community and has been proposed as a potential new therapeutic target and as a biomarker, in particular for cardiovascular diseases (CVD). However, many regulatory steps of circulating TMAO levels exist, making the picture complex and the correlation between TMAO metabolism and NCD still brought into question. This chapter describes the origin and the metabolism of TMAO, summarizes the current evidence about its association with major NCD, and describes potentialities and pitfalls of this gut-derived metabolite as a biomarker or therapeutic target in humans.

TMAO Metabolism: The Role of the Diet and the Microbiome

Dietary Sources

The competition between gut microbes and human cells for dietary ingredients derived from foods is at the basis of the complex interactions existing between foods, gut microbiota, and the host. Furthermore, the ability of the microbiome to transform dietary precursors in different metabolites (having positive or harmful effects) has a relevant role in the regulation of human health (Gentile and Weir 2018). TMAO metabolism should be thought into this context.

Circulating TMAO levels are affected by several factors, first among others, the diet. TMAO in its preformed state can be directly ingested from seafood, where it is present in high levels for osmoregulation (Ghaly et al. 2010). Moreover, choline, betaine, L-carnitine, dimethylglycine, and their precursors (e.g., phosphatidylcholine, crono-betaine, γ-butyrobetaine) can be converted into TMA by TMA-producing bacteria in the gut (Andraos et al. 2020).

Despite this direct relationship between diet, TMA and TMAO, conflicting findings about the dietary contributions to plasma TMAO concentrations have been reported (Wang et al. 2019; Bordoni et al. 2020a; Zhu et al. 2020). Several factors might contribute to different individual responses (in terms of circulating TMAO levels) to dietary TMA precursors. These include, among others, the gut microbiome composition (Cho et al. 2017), the genetic background of the host, and its excretion capacity.

TMA-Producing Bacteria

A major determinant of TMA and TMAO circulating levels is the gut microbiome composition. In particular, the abundance and activity of bacteria catalyzing TMA formation have a major role (Rath et al. 2018), since they compete with the host for the absorption of the dietary precursors. Bacteria produce TMA through two major pathways: the first has choline as a substrate and involves the choline TMA-lyase (CutC) and its activator choline trimethylamine-lyase activating enzyme (CutD); the second has carnitine and gamma-butyrobetaine as substrates and involves a two-component Rieske-type oxygenase/reductase (CntA/B). In addition, a third enzyme complex (YeaW/X), which shows close sequence similarity to CntA/B,

has been suggested as a key component of a third major pathway (Koeth et al. 2014). By analyzing the presence of the DNA sequences encoding for the mentioned enzymes in fecal samples, Rath and colleagues demonstrated that potentially TMA-producing bacteria are ubiquitously found in Mammalia (Rath et al. 2020), even though in a low abundance (<1.2% of total community). They also demonstrated that the composition of TMA-forming communities was influenced by the diet and the host taxonomy (Rath et al. 2020). For example, a reduced capacity to convert L-carnitine into TMA has been measured in subjects with vegan diet, suggesting that dietary habits can lead to a counterselection of L-carnitine metabolizing gut bacteria over time (Koeth et al. 2013). Thus, a bi-univocal relationship exists: the high inter-individual variation in the gut microbiome composition affects the TMA conversion of dietary precursors; but also, different amount of TMA dietary precursors shapes the gut microbiota composition.

Some studies described metabolic pathways leading to colon TMA production and attempted to define the bacterial strains responsible for this activity (Falony et al. 2015; Jameson et al. 2016). Findings suggest that TMA-producing bacteria encompass various species with members of *Enterobacteriaceae* as main contributors (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) (Fadhlaoui et al. 2020). CntA/B and YeaX/Y genes have been, respectively, found in several taxa from the *Gamma*- and *Betaproteobacteria* as well as from a few *Firmicutes* (Zhu et al. 2014), especially *Clostridium XIVa* (Rath et al. 2017). It appears that gut bacteria that can synthetize TMA are from different phylogenetic lineages, distributed across various taxa belonging to *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Martínez-del Campo et al. 2015), probably also because of lateral gene transfer phenomena. Falony et al. described how to define the TMA potential of the gut microbiome and provided a metagenomic characterization of an atherosclerosis-associated dysbiosis (Falony et al. 2015). Finally, it has recently emerged that TMA can be used for methanogenesis by specific archaea (Fadhlaoui et al. 2020); thus, TMA might not be an end-product in individuals who host these organisms.

TMAO Metabolism: From Absorption to Excretion

Most of TMA ingested or formed in the gut is rapidly absorbed by the human host by passive diffusion, conveyed into the liver by the portal circulation, and then oxidized to TMAO. This oxidation is mediated by specific hepatic enzymes: the flavin monooxygenases (FMO), especially FMO3 (Zeisel and Warrier 2017). For this reason, not only the diet but also the genetic background of the host can affect TMAO metabolism (Bennett et al. 2013). Impairment of the liver oxidation (such as in case of mutations in the FMO3 gene) (Dolphin et al. 1997) can reduce the conversion of TMA into TMAO (totally or partially), leading to increased circulating levels of TMA in the blood. Once entered in the circulation, the pharmacokinetics of TMA and TMAO in humans has been poorly investigated, although pharmacokinetics of chemically similar molecules has been described (Bain et al. 2005). A study using isotopically labeled TMAO (d9-TMAO) showed an early peak of d9-TMAO

in plasma (15 min after the intake) that increased until 1 h and remained elevated through the 6-h period. A fraction of TMAO can be taken up by extrahepatic tissues, at least by skeletal muscles (Taesuwan et al. 2017). In the blood, TMAO can interact with circulating proteins. Paul et al. demonstrated that TMAO can interact with a zinc protoporphyrin IX dimethyl ester [ZnPPDME] producing the complex [TMAOZnPPDME] that moves from the polar plasma to the nonpolar (lipid) site mediating the nonenzymatic cholesterol oxidation (Paul et al. 2021), which leads to 7-hydroxycholesterol (α and β), 5,6-epoxycholesterol (α and β), 7-ketocholesterol, and cholestane-3,5,6-triol. Overall, free TMAO in plasma is reduced, and cholesterol oxidation is increased not only by the classic oxidative mechanisms catalyzed by the cholesterol hydroxylase enzyme but also through this alternative nonenzymatic catalysis.

Additionally, TMA (but not TMAO) can interact with albumin and cardiac lactate dehydrogenase, leading to their degradation in 24 h (Jaworska et al. 2019b).

Furthermore, TMAO can act as a stabilizer of folded proteins (Ma et al. 2014); TMAO works on peptide hydration decreasing the strength of hydrogen bonds formed between protein polar groups and water; moreover, it increases protein stability as a molecular crowder, through the excluded volume effect. Besides, an interaction between TMAO and lipids has been described at plasma membrane level; TMAO reduces the head group hydration of the lipids increasing packing density of the membrane and affecting the orientation order of lipids (Maiti and Daschakraborty 2021). Thus, TMAO influences the fluid phase of membranes in a dose-dependent manner, and it can contrast the osmotic stress condition in deep-sea organisms.

Finally, TMAO generally has a high turnover and a fast clearance, being rapidly excreted by urines (Taesuwan et al. 2017). Also TMA, as a volatile molecule, can diffuse in body fluids and be excreted through sweat, breath, and urines (leading to trimethylaminuria or fish odor syndrome, in case of excessive circulating levels) (Dolphin et al. 1997).

TMAO and Diseases

TMAO Detection

TMAO has been studied, especially in humans, mainly in urine and in plasma samples, although it is sometimes measured in serum as well. In a few investigations, TMAO has been measured also in fecal waters, liver, muscle, brain, kidney, intestine, and cerebrospinal fluid (Zerbst-Boroffka et al. 2005; Laxson et al. 2011; Zhang et al. 2015; Del Rio et al. 2017; Wahlang et al. 2017).

Several methods have been implicated to this purpose. These include, among others, liquid chromatography-mass spectrometry, proton nuclear magnetic resonance spectrometry, headspace gas chromatography, stable isotope dilution high-performance liquid chromatography with electrospray ionization tandem mass spectrometry, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Janeiro et al. 2018).

TMAO and Cardiovascular Diseases

A body of evidence suggests that gut microbiome is implied in CVD pathogenesis (Mamic et al. 2020; Zhou et al. 2020). In 2011, Wang and colleagues firstly showed that choline, TMAO, and betaine (metabolites of dietary phosphatidylcholine) predicted the risk for CVD in an independent large clinical cohort (Wang et al. 2011). They also showed in a mouse model that dietary choline or TMAO supplementation promoted atherosclerosis and that the microbiome has an essential role in the production of TMAO.

From that time onward, a plenty of studies further investigated this association. Up to date, the link between TMAO and cardiovascular health has been confirmed by numerous findings (including systematic reviews and meta-analysis), and TMAO has been suggested as an independent predictor of CVD risk and mortality (Senthong et al. 2016; Heianza et al. 2017; Qi et al. 2018; Farhangi et al. 2020), with a dose-dependent effect (Schiattarella et al. 2017). A meta-analysis on 6879 patients demonstrated that TMAO levels are associated with a poor prognosis in patients with heart failure (Li et al. 2020). A prognostic value of TMAO has been suggested also by Hochstrasser and colleagues, showing that TMAO can predict short- and long-term mortality and poor neurological outcome in out-of-hospital cardiac arrest patients (Hochstrasser et al. 2020). Moreover, TMAO has been proposed as a biomarker for plaque rupture in patients with myocardial infarction (Yu et al. 2019), and it has been associated with increased odds of hypertension and reduced high-density lipoprotein cholesterol levels in apparently healthy individuals (Abbasalizad Farhangi and Vajdi 2020). Thus, all these findings suggest that TMAO has a potential usage both for early diagnosis and for prognosis of cardiovascular diseases.

Given the previously described TMAO metabolism, a major role of the microbiome in modulating this association has been confirmed. Metagenomic data-mining has revealed an enrichment of TMAO synthesis in gut microbiome in atrial fibrillation patients (Zuo et al. 2020). Moreover, specific gut microbial taxa have been demonstrated to successfully predict acute coronary syndrome and post-STEMI cardiovascular events in humans (Gao et al. 2020).

However, a definite mechanistic explanation for the association between TMAO and CVD onset is still lacking. Several hypotheses have been proposed; among others, the most quoted are the promotion of platelet aggregation (supported by evidence from human and mouse studies (Zhu et al. 2016, 2017)), the alteration of cholesterol homeostasis (Canyelles et al. 2018), the promotion of vascular inflammation (Sun et al. 2016), and the impairment of methyl metabolism (Obeid et al. 2016).

However, a major issue is to clarify if the increase of circulating TMAO is a cause or a consequence of the disease. In this regard, Drapala and colleagues recently showed disturbances of the gut-blood barrier in a spontaneously hypertensive heart failure rat model (Drapala et al. 2020). These included reduced intestinal blood flow, decreased thickness of the colonic mucosa, and alterations in tight junctions that were associated with increased TMA and TMAO levels in plasma (Drapala et al. 2020).

Finally, it has been hypothesized that not only TMAO but also TMA might play a role per se in CVD onset (Jaworska et al. 2019a; Drapala et al. 2020).

TMAO and Chronic Kidney Diseases

Since the kidneys filter out TMAO from the blood and excrete it through urine, it is not surprising that chronic kidney diseases (CKD) represent an important cofounder in TMA and TMAO association studies (Wu et al. 2019) and might affect circulating levels of these metabolites. Moreover, it has been shown that CKD directly alters the intestinal microbial flora (Anders et al. 2013; Vaziri et al. 2013). Both TMAO accumulation and its effect on the metabolism appear as a complex interplay between diet and microbiome on the one hand and system-level factors (such as circulating proteins, metabolites, and kidney function) on the other hand (Manor et al. 2018).

TMAO pathway has been suggested also as a biomarker for renal disease, with high levels of circulating TMAO detected in CKD patients. In addition, TMAO seems to actively contribute to the progression of renal disease and to the mortality risk in CKD (1.7-fold increase in risk for all-cause mortality in patients with high TMAO levels) (Fogelman 2015; Tang et al. 2015). Coherently, TMAO has been defined as an independent risk factor for hospitalization events in patients receiving maintenance hemodialysis (Zheng et al. 2020). Given that evidence, the tight relationship existing between renal function, cardiovascular health, and TMAO should be considered when investigating TMAO as a biomarker for these complex diseases.

TMAO and Metabolic Diseases

A body of literature has demonstrated that a complex relationship between gut dysbiosis and metabolic diseases exists (Pascale et al. 2018). The inflammatory status that is typical of an imbalanced gastrointestinal microbiome has been associated also with glucose metabolism alteration and even insulin resistance. This might be due to an altered production of several metabolites, including TMA and TMAO (Tanase et al. 2020). Indeed, there is convincing evidence suggesting an association between TMAO and inflammation (Rohrmann et al. 2016; Chen et al. 2017; Yue et al. 2017). Recently, Farhangi et al. also showed a nonlinear association between increased TMAO concentrations and increased C-reactive protein concentrations in a dose-response meta-analysis, corroborating the hypothesis of an association between TMAO and metabolic diseases also in humans (Farhangi and Vajdi 2020).

High TMAO has been detected in diabetic patients in a meta-analysis including 15,314 enrolled subjects, with an odd ratio for diabetes mellitus (DM) prevalence increased by 54% per 5 μmol L^{-1} increment of plasma TMAO (OR = 1.54) (Zhuang et al. 2019). Another similar meta-analysis identified a positive association between circulating TMAO and obesity, with a dose-dependent effect measured in apparently

healthy individuals (Dehghan et al. 2020). According to this hypothesis, Barrea and colleagues showed a correlation between TMAO, the Fatty Liver Index, a predictor of nonalcoholic fatty liver disease (NAFLD), and the visceral adiposity index, a gender-specific indicator of adipose dysfunction, suggesting a potential role of TMAO as an early predictor of metabolic syndrome (MetS) (Barrea et al. 2018).

TMAO and Other Diseases

TMAO has not only been linked to metabolic but also to other chronic or complex disease.

In particular, data on animal models suggest that TMAO could induce brain aging and age-related cognitive dysfunction (Li et al. 2018; Gao et al. 2019). It has been recently suggested that TMAO may induce cognitive deficits by promoting endoplasmic reticulum stress (Govindarajulu et al. 2020). Higher concentrations of TMAO in the cerebral spinal fluid have been observed in patients with mild cognitive impairment and Alzheimer's disease (AD) dementia (Vogt et al. 2018). However, a Mendelian randomization (MR) approach failed to demonstrate a causal effect between increased TMAO levels and AD in a total sample size of 455,258 patients and controls (Zhuang et al. 2020).

Variations in the concentrations of TMAO and its precursors have been measured also in patients with amyotrophic lateral sclerosis (Chen et al. 2020a).

Increased TMAO serum levels have been associated with high risk of hip fracture (Liu et al. 2020) and with functional impairment of bone marrow mesenchymal stem cells in osteoporosis disease (Lin et al. 2020).

TMAO as a Potential Therapeutic Target: From Nutrition Interventions to Gut Microbiota Modulations

The hypothesis to target TMAO metabolism to reduce the risk of NCD has been proposed. The targets could also be intermediary risk factors related to both TMAO and lifestyle (i.e., diet), such as hypertension (Naqvi et al. 2021). These goals could be achieved by regulating dietary intake of TMA precursors or by targeting the gut microbiome by nutritional interventions (He et al. 2020; Heng et al. 2020; Liang et al. 2020; Simó and García-Cañas 2020; Spence 2020; Wiese et al. 2020; Yu et al. 2020; Zhao et al. 2020) or supplements (Martin et al. 2008; Brugère et al. 2014). Potential pharmacological treatments have been also discussed (Dannenberg et al. 2020; Gencer et al. 2020; Steinke et al. 2020). However, several considerations should be made.

Pharmacological treatments have been firstly suggested to target TMA oxidation in the liver for reducing circulating TMAO levels. However, it should be considered that this inhibition might increase circulating levels of TMA, resulting in potentially harmful or side effects (i.e., trimetilaminuria). Another possibility is to use antibiotics to shape the gut microbiota in order to reduce the TMA production. However,

even if broad-spectrum antibiotics can be used to suppress the production of TMAO during a certain time of treatment, the TMAO production recovers after the removal of antibiotics (Wang et al. 2011; Tang et al. 2013).

Concerning dietary interventions, it should be considered that some of the TMA precursors are essential nutrients (i.e., choline) for humans. Thus, selecting foods that provide lower quantities of TMA precursors might affect the adequate intake of these essential nutrients. Since TMAO does not always increase after TMA precursors' ingestion (Zhu et al. 2020), it should be further investigated if this approach is beneficial or harmful for human health.

Interestingly, some foods or dietary approaches (reviewed by Janeiro et al. (2018)) might help to modulate TMAO circulating acting at different levels. For example, *Brassicaceae* vegetables can act on FMO3 by reducing its activity and, consequentially, TMA oxidation (Cashman et al. 1999; Janeiro et al. 2018). Garlic contains allicin that, through its antimicrobial property, might help the host to reduce TMAO formation from carnitine intake (Wu et al. 2015). Thus, food acting as functional modulators of gut microbiota might be applied to target circulating TMAO levels. Up to date, targeting the TMAO by pre- or probiotic supplementation or microbiome remodeling strategies (e.g., bioremediation of TMA by methanogenic archaea (Fadhlaoui et al. 2020)) appears as the most promising approach (Chen et al. 2020b), also considering that changes in the microbiome composition occur faster than changes in TMAO concentration. This could be applied not only for primary but also for secondary prevention interventions (Moludi et al. 2020).

Finally, recent findings showed that TMAO levels might be reduced also by increasing physical activity (Argyridou et al. 2020), suggesting an additional new mechanism underpinning the inverse relationship between physical activity and CVD and a new potential strategy for TMAO control.

TMAO as a Biomarker: Potentiality and Limits

Potentialities

If TMAO or TMA will be confirmed as valuable biomarkers, they might represent an easy accessible tool for early disease prediction and population risk stratification for major NCD that currently represent a big burden for the society. Thus, the early identification of individuals at risk for complex, multifactorial, and environmentally driven diseases is a current major objective of modern personalized medicine, with a significant predicted impact on population health. Moreover, especially in the case of CVD, these biomarkers might also have a prognostic value and be a support for the already available therapies. The usage of such biomarkers might be applied also for personalized primary and secondary prevention (targeting individual diet and microbiome composition).

However, several gaps in the knowledge on TMA and TMAO metabolism still represent major limits and obstacles to the translation of the usage of these biomarkers in the clinical practice.

Limits and Pitfalls

Despite that numerous studies, even recruiting big populations, detected significantly higher levels of TMAO in patients than in healthy individuals, most of them lack causal demonstration and mechanistic explanations of the detected association (Nowinski and Ufnal 2018).

Firstly, most of mechanistic findings about the pathogenic role of TMAO come from animal studies (Wang et al. 2011; Koeth et al. 2013). However, since resistance to atherosclerosis is a major limitation of mouse models, genetic modifications, such as low-density lipoprotein (LDL) receptor deficient (LDLR−/−) and apolipoprotein knockout (ApoE−/−), have been applied to induce hypercholesterolemia. While the association between TMAO and CVD was significant in ApoE−/− mice (Koeth et al. 2013), when they were transfected with human cholesteryl ester transfer protein, the association was reversed, and increased plasma TMAO was associated with a reduced area of aortic lesions (Collins et al. 2016). This evidence suggests that reverse cholesterol metabolism might mediate this association (Collins et al. 2016; Bordoni et al. 2020b).

Moreover, it has been recently demonstrated that circulating TMAO increases with fish consumption, but not according to red meat intake (Hamaya et al. 2020). In the same study, the authors show that unhealthful dietary patterns are inversely correlated to TMAO; on the contrary, a direct correlation is measured with healthy dietary patterns, suggesting that higher TMAO levels should not simply be interpreted as a marker of unhealthy dietary pattern or food intake. Since TMAO is present in seafood and fish consumption is considered to be beneficial for human health, all these findings suggest that the picture might be more complex than as initially hypothesized and the current paradigm might be revisited.

In accordance with this hypothesis, TMAO has been recently shown to have some protective effects in an animal model of hypertensive-heart-failure rats (Gawrys-Kopczynska et al. 2020), suggesting that the increase of TMAO might be a compensatory effect associated with a certain insult, rather than a cause (Papandreou et al. 2020). Indeed, a bidirectional Mendelian randomization approach has been recently applied to test causality between TMAO and CVD, but no significant associations of genetically predicted higher TMAO with CVD and related traits were measured (Jia et al. 2019).

Several confounding factors that might modulate the association between TMAO and NCDs exist. Firstly, a role of the genetic background in mediating the association between TMAO and NCDs has been hypothesized (Yazaki et al. 2020). Moreover, disturbances of the gut functions (i.e., gut inflammation, permeability, and dysbiosis (Drapala et al. 2020; Sánchez-Alcoholado et al. 2020)) might be major confounders in this association. In particular, how the microbiota composition affects TMAO levels and how it independently contributes to the risk for the chronic disease development should be elucidated before translating TMAO as a biomarker in clinical practice.

As previously mentioned, the biological fate of orally ingested TMA precursors can vary interpersonally because of the gut microbiota and may cause paradoxical biological effects in patients (Wu et al. 2019). To identify adequate personalized intake of carnitine, Wu et al. (2020) recently proposed an oral carnitine challenge test that could estimate TMAO productivity from carnitine metabolism in the human body. Further similar investigations are necessary to identify relevant TMAO producer phenotypes in both omnivores and vegetarians, for directing personalized advice on TMA precursor intake. Moreover, these studies might help to clarify inconsistent findings concerning the association between TMA precursor intake, TMAO levels, and NCDs.

In parallel, evidence suggests that disturbance of TMA metabolism might also be implied in CVD (Jaworska et al. 2019b; Bordoni et al. 2020b), making relevant to collect further data on TMA (and not only on TMAO) in all the previously mentioned disease conditions.

Finally, the pathological cutoff value of human plasma TMAO has not yet been clearly defined. Some indications in this regard can be found in current literature (Koeth et al. 2013; Tang et al. 2013, 2019; Schiattarella et al. 2017; Roberts et al. 2018; Wu et al. 2019), mainly in relation to CVD. In particular, Tang et al. suggested a TMAO level higher than 6.2 μM as a predictor for increased risk of cardiovascular event (Tang et al. 2013). Schiattarella and colleagues showed that relative risk for all-cause mortality increased by 7.6% per each 10 μmol/L increment of TMAO (Schiattarella et al. 2017). In vitro and animal studies (Koeth et al. 2013; Roberts et al. 2018) estimated that TMAO level for enhancing thrombosis potential was at least 10–30 μM, suggesting that plasma levels of TMAO higher than 10 μM in healthy subjects may increase the risk for CVD in the long term. However, further investigations to define cutoff values to be translated in clinical practice are necessary.

Conclusions

NCDs are a major burden for the modern society; thus adequate tools for population risk stratification are warranted. Moreover, since environmental factors, including diet, are major determinants of NCD, to monitor biological effect of dietary metabolites on human health is essential to set up efficient prevention strategies (i.e., nutritional intervention), especially in the emerging field of personalized nutrition (Wu et al. 2020). In this context TMAO appears as a promising candidate as an early risk and prognostic biomarker (Fig. 1). However, major doubts about mechanistic aspects and about clinical validity of this biomarker still exist (Kolluru Gopi and Kevil Christopher 2020). In particular, the role of other intermediaries involved in the TMAO metabolism (i.e., TMA and TMA-producing bacteria) should be clarified in order to exclude confoundings or reverse causality. For these reasons, further mechanistic investigations and validations are necessary before authorizing the translation of this biomarker in clinical practice.

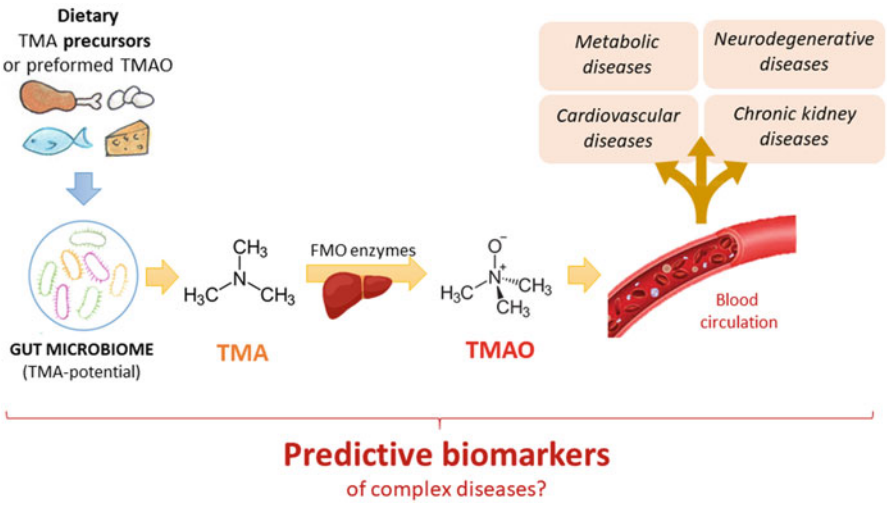

Fig. 1 TMA and TMAO metabolism. TMA is produced from dietary precursors by the gut microbiome. Through the blood circulation, it comes to the liver, and it is oxidized into TMAO, which has been associated with noncommunicable disease onset. Current researches are trying to demonstrate which among the intermediates of the TMA metabolism might be good predictive biomarkers of risk

Applications to Prognosis and Other Diseases or Conditions

Due to all the aspects described in this chapter, the usage of intermediates of the TMA metabolism for noncommunicable diseases prognosis is still under discussion, and a definite position of the scientific community is still missing. Up to now, these biomarkers have been defined in animal models and have been also tested in the adult population, which is the subgroup more at risk to develop noncommunicable diseases associated with environmental exposures. Only a few data have been collected in children (Andraos et al. 2020; Quan et al. 2020). Since it is not clear if the level of these metabolites is a cause or a consequence of the impaired homeostasis associated with the disease development, further studies on the gut microbiome composition and genetic background of each subject (including studies based on Mendelian randomization approaches) might help to demonstrate causality of this association opening the way to new therapies and preventive actions. However, a better understanding of molecular pathways modulated by these metabolites is necessary to translate this evidence into clinical practice, not only for CVD prevention (which is the research area that is currently investigating TMAO the most) but also for other disease conditions, especially neurodegenerative, metabolic, and kidney diseases.

Mini-Dictionary of Terms

- ***Gene knockout***: it is a permanent change in the DNA sequence of an organism, leading to the loss of function of a gene.
- ***Genome-wide association study (GWAS)***: GWAS is an approach used in genetics research to associate specific genetic variations with particular diseases. The method involves rapidly scanning several hundred thousand genetic markers across the genome of many people to find genetic variations associated with a specific trait.
- ***Mendelian randomization***: it is a methodological approach applied to avoid confounding or reverse causality. It uses human genetic variants as instrumental variables to allocate individuals in exposed/unexposed groups, analogously to a randomized controlled trial, where genetic alleles are randomly assorted during conception. The main objective of Mendelian randomization is to identify modifiable exposures that are valuable therapeutic targets and can be intervened on to improve health outcomes.
- ***Meta-analysis***: it is a statistical analysis that combines results from several scientific studies, providing a critical evaluation of the current evidence on a specific topic.
- ***Metagenomics***: is a molecular tool used to analyze DNA acquired from environmental samples, in order to study the community of microorganisms present without the requisite of obtaining pure cultures. An emerging field of application is the study of the gut microbiome, providing access to the functional gene composition of microbial communities and giving phylogenetic information, which can be based only on the diversity of one gene, such as the 16S rRNA gene.
- ***Noncommunicable disease (NCD)***: NCDs are chronic diseases, tend to be of long duration, and are the result of a combination of genetic, physiological, environmental, and behaviors factors. They include heart disease, stroke, cancer, diabetes, and chronic lung disease (Beaglehole et al. 2011).

Key Facts of Trimethylamine N-Oxide (TMAO) as a Biomarker: Features and Applications

Diet and microbiome composition regulate the TMA production in the gut.

TMA is absorbed and oxidized into TMAO, whose concentration has been associated with several NCDs.

The usage of TMA and TMAO as biomarkers appears to be a promising tool for the early identification of at-risk individuals and for targeted prevention intervention.

Mechanistic aspects explaining the role of TMA and TMAO in NCD pathogenesis are missing.

Further details about how TMA and a TMA-producing microbiome can lead to NCD will promote the translation of these biomarkers in the clinical practice.

Summary Points

- The origin of NCD is complex and multifactorial; diet and microbiome might play a role in their onset.
- Diet and microbiome composition regulate circulating levels of numerous metabolites that might affect human health; these include TMA and TMAO.
- TMAO has been associated with cardiovascular, metabolic, kidney diseases, neurodegeneration, and osteoporosis.
- TMAO has been proposed as a biomarker for disease prevention and prognosis. It might be used for population risk stratification, primary and secondary prevention, and personalized treatments.
- Mechanistic aspects that still have to be elucidated limit the application of this biomarker in clinical practice.

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The Redox State of Serum Albumin as a Potential Protein Nutrition Biomarker

3

Measures and Application

Yasuaki Wada

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Abstract

Albumin comprises the largest part of total serum proteins in humans and is exclusively synthesized in the liver, before being distributed into the circulation. This protein consists of 585 amino acid residues including a single free cysteine residue at position 34 (Cys34). The free thiol residue of the Cys34 is redox-active and specifies the redox state of serum albumin, termed mercaptalbumin (reduced albumin), non-mercaptalbumin-1 and -2 (oxidized albumin). The redox state of serum albumin has been viewed solely as a manifestation of systemic oxidative stress. However, a series of recent animal studies have shown the potential of

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serum albumin redox state as a novel protein nutrition biomarker, and this notion has been subsequently substantiated by an observational study of Japanese pregnant women. This chapter overviews the roles of serum albumin redox state in human health, with an emphasis on recent updates.

Keywords

Albumin · Biomarker · Cys34 · Mercaptalbumin · Non-mercaptalbumin · Pregnant women · Older adults · Protein undernutrition · Oxidative stress · Redox state of serum albumin

Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
BCAAs	Branched-chain amino acids
Cys	Cysteine
Cys34	Cysteine residue at position 34 in serum albumin
DRIs	Dietary reference intakes
FcRN	Neonatal Fc receptor
HNF-1	Hepatocyte nuclear factor 1
MA	Mercaptalbumin
mTOR	Mammalian target of rapamycin
NA	Non-mercaptalbumin
PLP	Pyridoxal 5′-phosphate
PMP	Pyridoxamine 5′-phosphate
PTB	Polypyrimidine tract-binding protein

Introduction

Protein-energy malnutrition is one of the major public health concerns for older adults, and is considered to be involved in aging-related diseases such as sarcopenia and frailty (Hernández Morante et al. 2019). In many countries, older adults apparently have sufficient protein intake as the intakes generally reach the estimated average requirements of dietary reference intakes (DRIs) (Tieland et al. 2015; Farsijani et al. 2016; Ishikawa-Takata and Takimoto 2018). However, the European Society for Clinical Nutrition and Metabolism recommends that 1.0–1.2 g/kg body weight is necessary for healthy older adults to maintain their skeletal muscle mass (Deutz et al. 2014), which is above the protein intake for considerable parts of the older population. Protein undernutrition is potentially but widely prevalent in this population, and biomarkers that indicate the insufficiency of dietary protein may help prevent these diseases. Serum protein concentrations are considered to reflect body protein pool, and the concentrations of albumin, transthyretin, transferrin, and retinol-binding have been measured as such biomarkers (Omran and Morley 2000). Albumin has a large circulating pool and its half-life is as long as 12–19 days, and the concentration is viewed as a reflection of protein nutritional status in the long term. On the other hand,

the half-lives of the other proteins, transthyretin, transferrin, and retinol-binding protein are quite shorter compared with albumin, and concentrations of these proteins have been considered to correlate more with acute changes of protein nutritional status. However, serum concentrations of these proteins are also modulated by factors other than protein nutritional status. Specifically, hepatic synthesis of these proteins is decreased by hepatitis, systemic inflammation, renal failure, aging, etc. (Omran and Morley 2000).

Among the above-mentioned serum proteins, post-translational modifications have extensively been investigated for serum albumin. Human serum albumin consists of 585 amino acid residues including 35 cysteine (Cys) residues (Tabata et al. 2021). Thirty-four Cys residues normally form intramolecular disulfide bridges and are involved in the "heart-shaped" tertiary structure (Quinlan et al. 2005), while the remaining single Cys residue at position 34 (Cys34) is free and redox-active (Fig. 1). The Cys34 provides the heterogeneity of three albumin isoforms, namely, mercaptalbumin (MA), non-mercaptalbumin-1 (NA-1), and NA-2, as extensively discussed later in this chapter. The redox state of serum albumin has been shown to be shifted to a more oxidized state in various pathological conditions such as hepatitis,

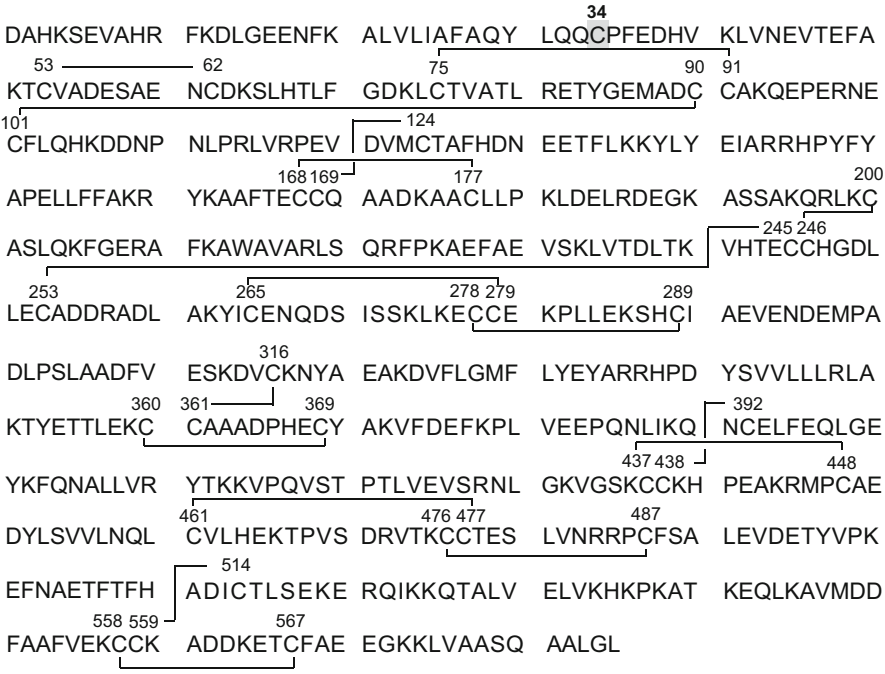

Fig. 1 Amino acid sequence of human serum albumin. The sequence is presented in single-letter code. Among the 585 amino acid residues, 34 of the Cys residues form disulfide bridges, while the remaining single Cys residue at position 34 (Cys34) is free and redox-active. Posttranslational modifications of the thiol group at Cys34 specifies the redox state of serum albumin. (This figure is adapted from Tabata et al. 2021, under the Creative Commons Attribution (CC BY 4.0) License)

renal failure, diabetes mellitus, and cardiovascular diseases, as well as physiological conditions such as aging and exercise training (Tabata et al. 2021). This has been mainly attributed to oxidative stress accompanying these conditions, and serum albumin oxidation has been viewed as a manifestation of systemic oxidative stress. Recently, a series of animal studies have unraveled a new potential of serum albumin redox state. Namely, the redox state of serum albumin was associated with albumin turnover and was sensitively responsive to dietary protein in rats (Wada et al. 2017; Wada et al. 2018; Wada et al. 2020). Hence the redox state of serum albumin would be useful as a novel protein nutrition in humans.

This chapter overviews serum albumin and its redox state, before featuring potential usefulness of serum albumin redox state as a sensitive protein nutrition biomarker.

Biological Roles of Serum Albumin

Human serum albumin has a single polypeptide chain of 585 amino acid residue as described above, and the theoretical molecular weight is 66,438 kDa. It is exclusively synthesized in the liver, and then secreted into blood circulation. Albumin is distributed in the intravascular (i.e., plasma) and extravascular compartments in a ratio of 3:7 to 4:6 (Watanabe et al. 2017). Still, albumin concentration is the highest among the serum proteins (approx. 0.6 mM), corresponding to 60–65% of the total serum proteins (Gatta et al. 2012). Because of this abundance, serum albumin contributes to the provision of colloidal osmotic pressure in the circulation, comprising as high as approx. 80% of the total pressure (Michelis et al. 2016). Notably, hepatic albumin synthesis receives feedback regulation by colloidal osmotic pressure, contributing to its homeostasis (Pietrangelo et al. 1992; Yamauchi et al. 1992). Another important biological role of serum albumin is to serve as a carrier of various kinds of endogenous ligands such as long-chain fatty acids, bilirubin, metal ions, and exogenous ligands such as warfarin and ibuprofen (Hoogenboezem and Duvall 2018). Moreover, serum albumin exerts an antioxidative activity mediated by the free thiol group of Cys34. This thiol group scavenges various kinds of reactive oxygen and nitrogen species such as hydrogen peroxide, peroxynitrite, superoxide, and hypochlorous acid (Roche et al. 2008; Anraku et al. 2013), which helps maintain the redox homeostasis in the circulation. Lastly, due to the large pool and long half-life in the circulation, serum albumin is considered to function as a reservoir of excessive dietary amino acids that is preserved from irreversible oxidation (Visser et al. 2005), although it has been suggested by some studies that the contribution of serum albumin as a reservoir might be limited compared with the skeletal muscle (Moore et al. 2009). Thus, serum ALB have multiple biological roles in human health.

Albumin Synthesis and Breakdown

Albumin is exclusively synthesized in the liver as described above, and albumin gene expression is primarily regulated by a transcription factor, hepatocyte nuclear factor 1 (HNF-1) (Wu et al. 1994). HNF-1 binds to the promoter of albumin gene and

induces the expression. This transcription factor is involved in the feedback regulation of albumin synthesis in response to colloidal osmotic pressure. Specifically, the binding affinity of HNF-1 to albumin gene was decreased in hepatoma cells in the presence of albumin (Pietrangelo and Shafritz 1994), and albumin gene expression was suppressed in rats when albumin was intravenously administered (Pietrangelo et al. 1992). In addition to colloidal osmotic pressure, the binding of HNF-1 to the albumin gene promoter is also modulated by nutritional factors. A decrease in the binding affinity was observed in rats when they were maintained in an amino acid-depleted condition (Oka et al. 1997). Pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) are the active forms of vitamin B6, and the hepatic PLP/PMP ratio was increased in the amino acid-depleted condition due to decreased conversion from PLP to PMP mediated by transamination (Oka et al. 1997). PLP but not PMP formed a complex with HNF-1, and this inhibited the binding of HNF-1 to albumin gene promoter and attenuated the gene expression (Oka et al. 2001). Thus, albumin gene expression is influenced by both colloidal osmotic pressure and amino acid/protein nutritional status, which is mediated by HNF-1 and/or PLP. Transcriptional regulations of albumin gene expression are summarized in Fig. 2.

Albumin synthesis is also regulated at the translational level in the nutritional context. The level of a eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) was increased in the liver of rats maintained even in a moderately protein-insufficient condition, with no change in the phosphorylation status (Kuwahata et al. 2018; Wada et al. 2020). This attenuated global protein translation including albumin translation. In addition, in a rat model of liver injury, polypyrimidine tract-binding protein (PTB) was bound to albumin mRNA, and the complex inhibited the access of ribosome to albumin mRNA, thereby suppressing albumin translation (Kuwahata et al. 2004). This suppression was then alleviated by branched-chain amino acid (BCAA) supplementation. According to a subsequent

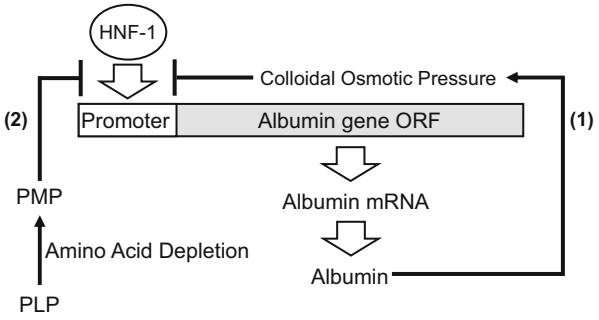

Fig. 2 Regulation of albumin gene transcription. Albumin gene is exclusively expressed in the liver, and the gene expression is primarily regulated by a transcription factor, hepatocyte nuclear factor 1 (HNF-1). Albumin gene expression is induced by the binding of HNF-1 to the gene promoter, while this transcription factor receives a feedback regulation in response to colloidal osmotic pressure (1). In addition, in an amino acid-depleted condition, the ratio of pyridoxal 5′-phosphate (PLP) to pyridoxamine 5′-phosphate (PMP) is increased, and PLP formed a complex with HNF-1, thereby decreasing the binding affinity of HNF-1 to albumin gene promoter and attenuating the gene expression (2)

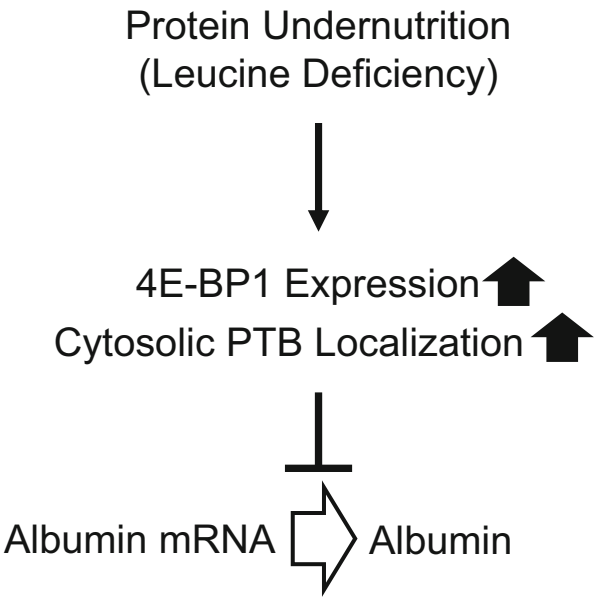

Fig. 3 Translational regulation of albumin synthesis. In a protein insufficient condition, the level of a eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is increased in the liver, with no change in the phosphorylation status; this attenuates global protein translation including albumin translation. In addition, in an amino acid deficient condition (especially leucine deficient condition), polypyrimidine tract-binding protein (PTB) is localized from nucleus to cytoplasm and forms a complex with albumin mRNA. This inhibits the access of ribosome to albumin mRNA, thereby suppressing albumin translation

hepatoma cell assay, PTB was localized from nucleus to cytoplasm and formed a complex with albumin mRNA in an amino acid-deficient condition, whereas the situation was reversed in an amino acid-sufficient condition (Kuwahata et al. 2008). Among the amino acids tested, leucine was found to be responsible for specifying the intracellular localization of PTB, which was mediated by mammalian target rapamycin (mTOR) signaling pathway. Although it was only revealed in rat models and a hepatoma cell assay, hepatic albumin synthesis is thus regulated at the translational level mediated by amino acid/protein nutritional status. Translational regulations of albumin synthesis are summarized in Fig. 3.

In contrast with albumin synthesis, albumin breakdown has less been elucidated to date. It has been considered that albumin breakdown occurs in all tissues, especially in muscle, liver, and kidney (Quinlan et al. 2005). Albumin is initially taken up from circulation by pinocytosis of the tissue cells, enters endosomes, and then receive lysosomal degradation. A variety of albumin binding proteins are considered to be involved in this catabolic process, and the neonatal Fc receptor (FcRn) is one of the receptors that have been well characterized (Bern et al. 2015). FcRN is predominantly localized to acidified endosomes, where it binds to albumin and sort albumin back to the circulation for recycling. In mice whose FcRN gene was conditionally deleted in the endothelial and hematopoietic cells, the serum albumin concentration was

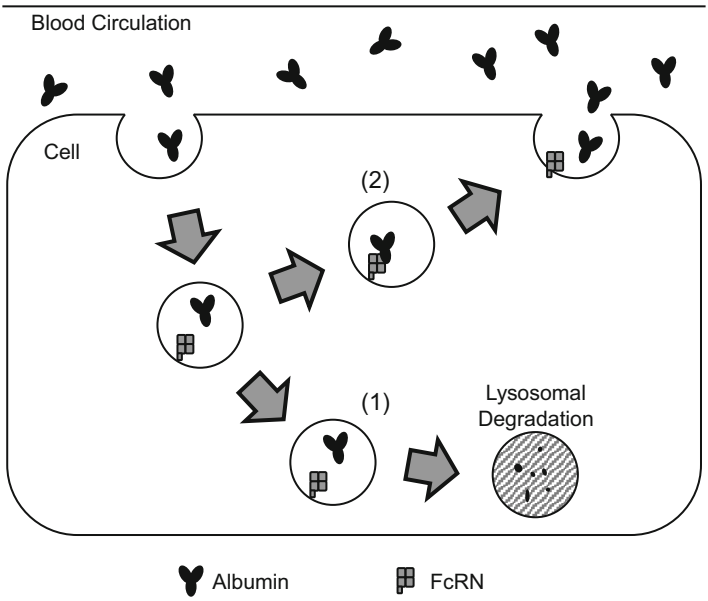

Fig. 4 Regulation of albumin breakdown. Albumin breakdown is considered to occur in all tissues, especially in muscle, liver, and kidney. Albumin is initially taken up from circulation by pinocytosis of the tissue cells, enters endosomes, and then receive lysosomal degradation (1). However, the neonatal Fc receptor (FcRn), which is predominantly localized to acidified endosomes, binds to albumin and sorts albumin back to the circulation for recycling (2). In a severely dietary protein-restricted condition where hypoalbuminemia is evident, the binding of FcRN to albumin is unsaturated and albumin is sorted more to recycling. This extends the half-life of albumin in the circulation

approximately twofold lower compared with the control animal (Montoyo et al. 2009), supporting the notion that FcRN rescues albumin from lysosomal degradation. On the other hand, albumin catabolic rate was reduced and its half-life extended in a severely dietary protein-restricted condition in humans and rats (Jeffay and Winzler 1958; James and Hay 1968). This can be interpreted as that the binding of FcRN to albumin was unsaturated and albumin was sorted more to recycling. This interpretation was substantiated by the observation in Nagase "analbuminemic" rats that the half-life of intravenously injected albumin was approx. 2.2 times longer compared with wild type rats (Esumi et al. 1979). Thus, FcRN suppresses albumin catabolism in an amino acid/protein-insufficient condition, and helps prevent a decrease in serum albumin concentration. The regulation of albumin breakdown is depicted in Fig. 4.

Redox State of Serum Albumin

As already described above, the redox state of Cys34 is involved in heterogeneity of serum albumin isoforms (Fig. 5). The isoform with the reduced thiol group at Cys34 is termed MA, which comprise 70–80% of total albumin in healthy young subjects (Oettl and Marsche 2010). On the other hand, the thiol group of Cys34 receives a disulfide

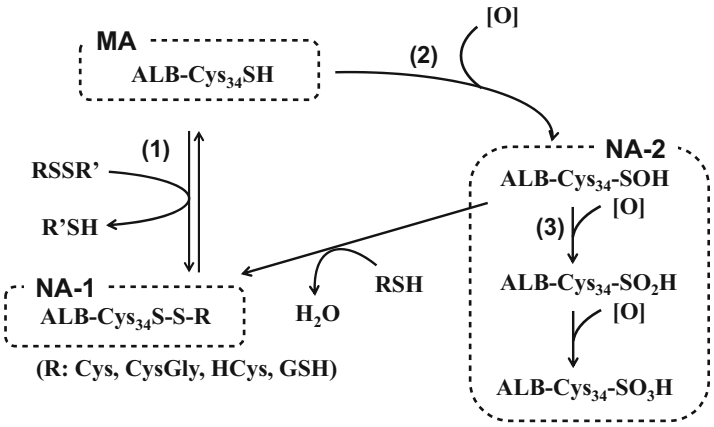

Fig. 5 Redox interplay between serum albumin and low-molecular-weight compounds. A disulphide exchange reaction occurs between the free thiol group in cysteine residue at position 34 (Cys34) of human serum mercaptalbumin (MA) with low-molecular-weight disulfides consisting of cysteine (Cys), cysteinylglycine (CysGly), homocysteine (HCys), and/or glutathione (GSH), to form a mixed disulfide on Cys34 (1); the resulting oxidized albumin is termed non-mercaptalbumin-1 (NA-1). Cys34 also reacts with several reactive oxygen/nitrogen species; the thiol group is initially oxidized to sulfenic acid (2), and is further oxidized to sulfinic and sulfonic acid (3). The resulting oxidized albumin is termed NA-2. (This figure is adapted from Tabata et al. 2021, under the Creative Commons Attribution (CC BY 4.0) License)

exchange reaction with low-molecular-weight disulfides consisting of Cys, cysteinylglycine, homocysteine, and/or glutathione. This forms a mixed disulfide at the Cys34, and the resulting oxidized isoform of albumin is termed NA-1. The thiol group of Cys34 also reacts with reactive oxygen/nitrogen species, oxidized initially to sulfenic acid and further to sulfinic and sulfonic acid, and the resulting oxidized albumin isoform is termed NA-2. These oxidized serum albumin isoforms, NA-1 and NA-2, account for 20–30% and 2–5% of total ALB, respectively, in healthy young subjects (Oettl and Marsche 2010). These oxidations modulate various kinds of biological properties of serum albumin. Initially, serum albumin is the dominant antioxidant in the circulation as described above (Anraku et al. 2013), but the anti-oxidative potential is impaired in oxidized albumin (Brioschi et al. 2020). Secondly, serum albumin dominantly contributes to the colloidal osmotic pressure as described above (Pietrangelo et al. 1992; Yamauchi et al. 1992), but the contribution is larger for oxidized albumin compared with reduced albumin (Michelis et al. 2016). Thirdly, serum albumin is a carrier of various endogenous and exogenous ligands, but the binding affinities are different between reduced and oxidized albumin isoforms. Specifically, binding affinities for endogenous ligands, bilirubin and tryptophan, as well as those for exogenous drug substances, warfarin and diazepam, are attenuated by albumin oxidation (Nagumo et al. 2014). Besides, proatherosclerotic lipids, lysophosphatidylcholine and lysophosphatidic acid show higher affinities for oxidized albumin, whereas anti-atherosclerotic mediators derived from eicosapentaenoic acid and docosahexaenoic acid exhibits higher affinities for reduced ALB isoform (Kurano et al. 2019).

Analytical Chemistry for the Redox State of Serum Albumin

These three albumin isoforms can be separated chromatographically, and their ratios have been frequently determined by HPLC in combination with spectrometric detection. Specifically, an anion-exchange column harboring diethylaminoethyl as a functional group, and the elution by a 40-min gradient of increasing EtOH concentration in the solvent of 0.4 M Na_2SO_4 and 0.05 M CH_3COONa (pH 4.85) have been commonly selected for the determination of serum albumin redox state (Hayashi et al. 2000). Yasukawa et al. recently developed a new system for analyzing the redox state of serum albumin using HPLC, where an in-house column of anion-exchange gel prepared from polyvinyl alcohol cross-linked gel reacted with diethylamine, and the elution by two kinds of phosphoric acid buffers makes it possible to determine the ratios of albumin isoforms as quick as in 9 min (Yasukawa et al. 2018). For detection, frequently used are measurements of UV absorbance at 215 nm derived from peptide bonds in the protein and fluorescence emission of a tryptophan residue at position 214 in human serum albumin (280 nm for excitation and 340 nm for emission). However, these spectrometric measurements are confounded by metabolites such as uric acid (for UV absorbance measurement) and bilirubin (for fluorescence emission measurement). Ueyama et al. circumvented this issue by derivatization of serum albumin with bromocresol green after chromatographic separation (Ueyama et al. 2015). Detection of derivatized albumin is made by UV absorbance at 620 nm and hence avoids the interference of the above metabolites.

Recent emergence of high-resolution MS technology has greatly contributed to the characterization of post-translational modifications in oxidized albumin. Post-translational modifications in oxidized albumin have been commonly characterized by subjecting "intact" albumin to MS in order to determine the "mass shifts" between reduced and oxidized albumin isoforms. Post-translational modifications (and mass shifts) that have been deduced by MS analyses include N-terminal loss of asparagil-alanine residue(−186), C-terminal of leucine residue(−113), cysteinylation (+119), homocysteinylation (+133), glycation (+162), sulfinylation (+32), and sulfonylation (+48) (Das et al. 2017; Domenicali et al. 2014; Leblanc et al. 2018). However, the MS resolution of analyzing intact serum albumin might be insufficient in general, casting doubt on the accuracy of post-translational modifications deduced by this method. On the other hand, bottom-up proteomics is the method that makes it possible to characterize post-translational modifications in proteins of with high reliability. It also enables site-specific identification of post-translational modifications in proteins (Aebersold and Mann 2003). Proteins of interest are digested using sequence-grade digestive enzymes such as trypsin and serine protease Glu-C prior to LC-MS/MS analysis, and spectra of ~5–20 amino acid-long peptides are normally detected with charge states +2–4. The subsequent assignment of these spectra to peptide sequences with post-translational modifications are executed with the match tolerance set to ~10 ppm. Use of the bottom-up proteomics, cysteinylation, homocysteinylation, sulfinylation on Cys34 have been observed (Nakashima et al. 2018). Still the application of bottom-up proteomics to the characterization of post-translational modifications in serum albumin is insufficient, warranting further investigation.

Association Between the Redox State of Serum Albumin and Pathological Conditions

The redox state of serum albumin has been extensively documented in the association with various kinds of diseases including hepatitis, renal failure, diabetes mellitus, and cardiovascular diseases (Tabata et al. 2021). Generally, the redox state of serum albumin shifts to a more oxidized state in patients compared with healthy controls, and the shift increases in proportion to the severity of pathological conditions. In liver disease patients, an increase in the level of NA-1 has generally been observed (Fukushima et al. 2007; Domenicali et al. 2014; Stauber et al. 2014; Das et al. 2017; Setoyama et al. 2017; Alcaraz-Quiles et al. 2018), whereas the increased level of NA-2 has also been confirmed especially in some types of the hepatitis (Stauber et al. 2014; Das et al. 2017; Alcaraz-Quiles et al. 2018). Branched-chain amino acids (BCAAs) are deficient in liver patients, and dietary BCAA supplementation is routinely selected for the treatment of hypoalbuminemia seen in these patients (Kurpad et al. 2006). Notably, this supplementation has been reported to reverse the oxidized shift of serum albumin redox state (Setoyama et al. 2017; Fukushima et al. 2007), which can be explained by the ideas that the BCAA supplementation would (1) replenish the BCAAs in the body as substances for ALB synthesis, and (2) stimulate protein synthesis including albumin through the activation of mTOR signaling pathway, leading to the increased influx of reduced albumin. In patients with renal failure, the oxidized shift of serum albumin redox state has been mainly recognized as an increase in the sum of oxidized albumin isoforms (NA-1 + NA-2) (Nakatani et al. 2018; Kobayashi et al. 2020), but the increased level of cysteinylated albumin (i.e., NA-1) has been confirmed in some studies (Regazzoni et al. 2013; Nagumo et al. 2014). Hemodialysis is a treatment for renal patients that removes waste metabolites such as creatinine and urea from the circulation. Notably, this treatment has been reported to reverse the oxidized shift of serum ALB redox state (Regazzoni et al. 2013); the mechanism remains an open question but the involvement of reducing agents in dialysis membranes is implicated. In the case of diabetes mellitus and cardiovascular diseases, sobering observations are that the extent of oxidized shift of serum albumin redox state has been reported to correlate with the loss of their "quality of life," i.e., activities of daily living for diabetic patients (Fukuhara et al. 2020), and cardiopulmonary exercise capacity for patients with cardiovascular diseases (Brioschi et al. 2020).

Even though these oxidized shifts of serum albumin redox state have been widely viewed as the manifestation of systemic oxidative stress in these pathological conditions, a new interpretation is currently emerging: oxidized serum albumin per se is a potential factor that aggravates the pathological conditions of various kinds of diseases. Specifically, fractions of NA-1 and NA-2 obtained from serum of liver patients elicited a cytokine storm in leucocytes and a respiratory burst of neutrophils, respectively (Das et al. 2017; Alcaraz-Quiles et al. 2018). Similarly, serum albumin fractions obtained from renal patients induced platelet aggregation and promoted the production of a pro-inflammatory cytokine IL-6 in human umbilical endothelial cells (Pasterk et al. 2016; Magzal et al. 2017). Furthermore, the study by Inoue et al. is

notable as they reported that oxidized serum albumin facilitated cancer metastasis in cell assays and animal models (Inoue et al. 2018). A cohort study in patients with head and neck squamous cell carcinomas then substantiated the above observations, reporting that patients with higher serum oxidized ALB levels exhibited higher plasma levels of neutrophil extracellular trap, and higher incidences pulmonary cancer metastasis (Inoue et al. 2018). In collection, these new interpretations of serum albumin redox state require further extensive investigation in order to elucidate its clinical significance.

Association Between the Redox State of Serum Albumin and Physiological Conditions

The redox state of serum albumin is also shifted by non-pathological conditions such as aging and exercise training (Tabata et al. 2021). Both free thiols and protein thiols in serum are generally converted to disulfides with aging (Giustarini et al. 2006), and an age-related increase in oxidized albumin has been confirmed in both pathological and non-pathological conditions (Kobayashi et al. 2020; Ueno et al. 2020). Cysteinylation and homocysteinylation are likely responsible for the aging-related increase in oxidized albumin (Rossi et al. 2009), and these observations have been interpreted as the manifestation of aging-related oxidative stress. As for exercise training, an oxidized shift of serum albumin redox state has initially been reported for the training of Kendo, a Japanese martial art fencing, in university elite athletes (Imai et al. 2002), where both NA-1 and NA-2 ratios significantly increased after a 5-day training camp compared with the ratios before the camp. A similar observation was reported in a special anti-terrorism force of Austrian police; the ratio of HNA-1 but not of HNA-2 increased after the performance of a cycle ergometer at 70–80% VO_{2max}, which was in proportion to the exercise intensity (Lamprecht et al. 2008). These two research groups also explored effectiveness of dietary antioxidant supplementation on reversing the oxidized shifts of serum albumin redox state. The intake of propolis, a plant resin collected by honey bee, mitigated the oxidized shift of serum albumin redox state by a kendo training camp (Imai et al. 2005), whereas ingestion of encapsulated juice powder concentrate showed no effect on the oxidized shift of serum albumin redox state by the ergometer training (Lamprecht et al. 2009). Finally, the report by Ashikawa et al. is notable as they indicated the association between the redox state of serum albumin and the quality of life, i.e., exercise capacity in older adults (Ashikawa et al. 2020).

Redox State of Serum Albumin as a Potential Protein Nutrition Biomarker

Serum albumin concentration has long been used as a protein nutrition biomarker, although it is now regarded more as a risk factor of morbidity and/or mortality in pathological conditions (Gatta et al. 2012). In contrast with serum albumin

concentration, the redox state of serum albumin was not examined in the context of nutrition until recently. An exception was the finding that oral BCAA supplementation reversed the oxidized shift of serum albumin redox state in liver patients (Fukushima et al. 2007; Setoyama et al. 2017), which would be mediated by improved de novo reduced albumin synthesis. It was therefore speculated that the redox state of serum albumin might correlate with albumin turnover that is modulated by amino acid/protein nutritional status. To test this hypothesis, a series of animal experiments were conducted to examine the associations between protein nutritional status, albumin turnover, and the redox state of serum/plasma albumin.

Initially, Kuwahata et al. examined effects of dietary energy and protein on the redox state of plasma albumin in growing rats, and it was found that the redox state of plasma albumin was shifted to a more oxidized state by protein-energy restriction or low-protein diet ingestion (Kuwahata et al. 2017). A subsequent study by Wada et al. demonstrated that the oxidized shift of plasma albumin redox state in growing rats was dependent on amount of protein intake but independent of energy intake (Wada et al. 2017). The redox state of plasma albumin was more responsive to dietary protein insufficiency compared with plasma albumin concentration, and the oxidized shift was exclusively to an increase in the ratio of NA-1. This shift was irrelevant to oxidative stress as determined by plasma concentrations of thiobarbituric acid reactive substance and advanced oxidation protein products. Notably, plasma MA ratio of the growing rats correlated with their albumin fractional synthesis rates in the liver. Besides, although not examined in that study, the half-life of plasma albumin was speculated to be extended in this severely protein undernourished condition accompanied by hypoalbuminemia as discussed above (Jeffay and Winzler 1958; James and Hay 1968). The extent of albumin oxidation would be increased by longer period of retention in the circulation (Kubota et al. 2009; Colombo et al. 2012). Thus, these observations collectively supported the above hypothesis that amino acid/protein nutritional status would modulate albumin turnover, thereby shifting serum/plasma albumin redox state. More specifically, protein undernutrition leads to an oxidized shift of serum albumin redox state, possibly by (1) decreasing albumin synthesis rate and lowering de novo reduced albumin synthesis the liver, and (2) extending the half-life of serum/plasma albumin and increasing albumin oxidation in the circulation.

Dietary protein "quantity" was found to be involved in modulating the redox state of plasma albumin as above, while an involvement of dietary protein "quality" was examined in the following studies. Kuwahata et al. maintained growing rats on one of the three low-protein diets containing the same amount of casein, egg white, or wheat gluten, and it was found that the oxidized shift serum albumin redox state was greater for the casein-based low-protein diet group compared with the two low-protein diet groups (Kuwahata et al. 2018). Sulfur amino acids are limiting amino acids for growth and maintenance of rats placed on casein (Reeves et al. 1993), and supplementation cystine to the casein-based low-protein diet group mitigated the oxidized shift of serum albumin redox state. These results suggested that the redox state of plasma albumin would be influenced by amino acid balance in the dietary protein, i.e., a part of dietary protein quality. Wada et al. then conducted a study similar to this (Wada et al. 2019); notably, this study examined supplementation with cysteine in the form of

glutathione. It was found that glutathione supplementation only reversed the oxidized shift of plasma albumin redox state by the low-protein diet ingestion to an extent similar to cystine supplementation or supplementation with the constituting amino acid mixture of glutathione (i.e., glutamic acid, cystine, and glycine). This observation implied that glutathione would be utilized merely as a source of cysteine for de novo albumin synthesis rather than serve as an antioxidant in this animal model.

Although the association between the quality and quantity of dietary protein and the redox state of plasma albumin was thus extensively investigated using growing rats as above, these animal models have high dietary protein requirements and are therefore quite sensitive to dietary protein intake, which raised the concern that the observations in these models could be only extrapolated to limited situations of human nutrition. To address this, Wada et al. examined another animal model that simulated "potential" protein undernutrition (Wada et al. 2018; Wada et al. 2020). Namely, adults rats were used instead of growing rats, as they have a lower dietary protein requirement and are less responsive to low-protein diet ingestion (Reeves et al. 1993). Body weights were not different between the low-protein diet group and the control group, and the differences in plasma concentrations of albumin and transthyretin were only seen in limited time points of the experimental period. In contrast, the oxidized shift of plasma albumin redox state in the low-protein diet group was significant and constant throughout the experimental period. Thus, a "potentially" protein-undernourished condition was simulated in that model, and the redox state of plasma albumin was the only parameter that was sensitive enough to indicate the potentially protein-undernourished status. It is hence considered that the redox state of serum/plasma albumin could be useful as a novel protein nutrition biomarker in humans. The relationship between protein undernutrition and the redox state of serum/plasma albumin elucidated is depicted in Fig. 6.

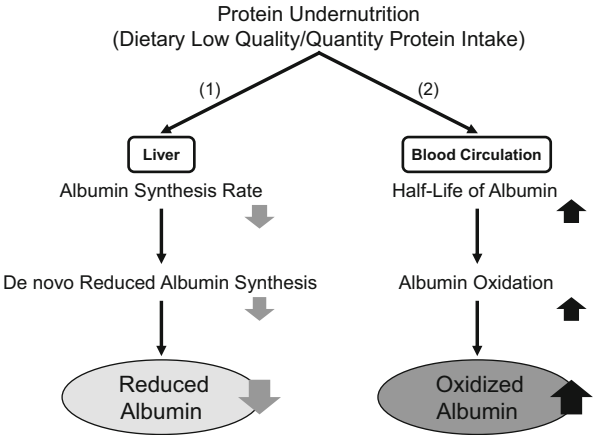

Fig. 6 The relationship between protein undernutrition and the redox state of serum/plasma albumin. Protein undernutrition (dietary low quality/quantity protein intake) leads to an oxidized shift of serum albumin redox state mediated by decreasing albumin synthesis rate and lowering de novo reduced albumin synthesis in the liver (1), and by extending the half-life of albumin and increasing the extent of albumin oxidation in the circulation (2)

Application of the Redox State of Serum Albumin to Prognosis of Protein Nutritional Status in Humans

Although prevalence of protein undernutrition has been a great concern among older adults, the usefulness of the redox state of serum albumin as a protein nutrition biomarker has not been investigated to date in this population. Still, Wada et al. recently examined the association between the redox state of serum albumin in Japanese pregnant women and their infant birthweight (Wada et al. 2021). The background of this study was that the ratio of low birthweight delivery is clearly higher in Japan (9.4%) compared with the average of Organization for Economic Cooperation and Development countries (6.5%) (Nomura et al. 2019), which may derive from potentially undernourished status prevalent among Japanese women at a reproductive age. Specifically, desire for thinness has been rooted in this population (Hayashi et al. 2007), and this could be responsible for the facts that the mean energy and protein intakes of Japanese pregnant women did not reach the requirements of the Japanese DRIs (Kubota et al. 2013; Kitamura et al. 2017). Wada et al. initially examined the relationship between three serum parameters, i.e., reduce albumin ratio, albumin concentration, and blood urea nitrogen concentration vs infant birthweight in 229 Japanese pregnant women (Wada et al. 2021). It was found that serum reduced albumin ratio in the third trimester was the only parameter that significantly correlated with infant birthweight. As a dietary assessment was not conducted in this observational study, Wada et al. also conducted an animal experiment simulating fetal growth restriction by maintain pregnant rats (dams) on graded levels of protein-energy restriction. It was observed that protein-energy restricted dams showed lower serum reduced albumin ratios and lower birthweights of the offspring compared with the control animals. Besides, serum albumin reduced albumin ratio in late-pregnancy correlated with birthweights of the offspring, as seen in the human observational study. In collection, maternal serum reduced ALB ratio in the third trimester is associated with infant birthweight in Japanese pregnant women, which would be mediated by maternal protein nutritional status as indicated by a fetal growth restriction model in rats. Although further clinical studies involving dietary assessment and/or dietary intervention are required, this is the first study that demonstrates the usefulness of serum albumin redox state as a protein nutrition biomarker in humans.

Conclusions

The redox state of serum albumin has long been interpreted only in the context of oxidative stress, but the interpretation has changed dramatically during the past decade (Fig. 7). Recent ex vivo studies have unraveled that oxidized form of albumin per se could elicit inflammation and aggravate pathological conditions. Moreover, a series of animal studies have shown the potential usefulness of serum albumin redox state as a sensitive protein nutrition biomarker, and this has been substantiated by a recent observational study of Japanese pregnant women. This potential protein

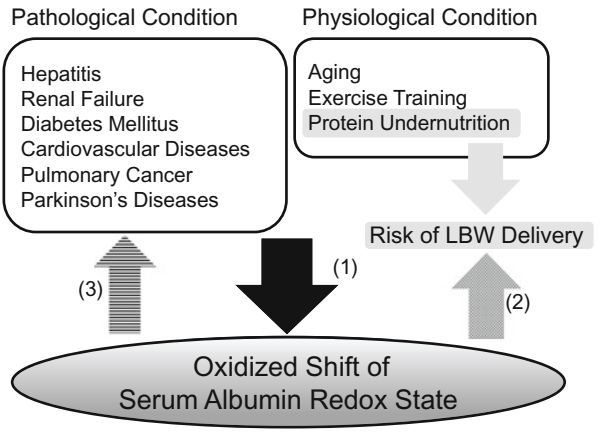

Fig. 7 Association between the redox state of serum albumin and pathological and physiological conditions. The redox state of serum albumin shifts to a more oxidized state in pathological conditions such as hepatitis, renal failure, diabetes mellitus, and cardiovascular diseases, and physiological conditions such as aging, exercise training, and protein undernutrition (1). Notably, maternal serum albumin redox state has shown a correlation with infant birthweight in Japanese pregnant women, suggesting that it would indicate the risk of low birthweight (LBW) delivery brought about by maternal protein undernutrition (2). Recent ex vivo studies further have shown the possibility that oxidized serum albumin per se could aggravate the pathological conditions (3). (This figure is adapted from Tabata et al. 2021, under the Creative Commons Attribution (CC BY 4.0) License)

nutrition biomarker could be also applicable to the assessment of protein undernutrition especially in older population, and clinical studies examining this is warranted as a future direction.

Mini-dictionary of Terms

- **Albumin**: The most abundant protein in the circulation. Serum albumin concentration has been classically used as a protein nutrition biomarker in clinical sites.
- **Cys34**: The abrreviation for the cysteine residue at position 34 in serum albumin. It has a free and redox-active thiol group, which specifies the redox state of serum albumin.
- **Mercaptalbumin**: The reduced form of albumin, which has a free thiol group at Cys34.
- **Non-mercaptalbumin-1**: One of the oxidized forms of albumin, having a mixed disulfide at Cys34 with low-molecular-weight thiols such as cysteine, homocysteine, and glutathione.
- **Non-mercaptalbumin-2**: The other oxidized forms of albumin, with the thiol group of Cys34 oxidized to sulfinic or sulfonic acid.
- **Redox state of serum albumin**: Alternatively, serum albumin redox state, which refers to the ratio of reduced and oxidized forms of serum albumin. This has long

been viewed as a manifestation of systemic oxidative stress. However, it has been recently shown to correlate with albumin turnover, and the usefulness as a sensitive protein nutrition biomarker is suggested.

Key Facts of the Redox State of Serum Albumin

- The biological interpretation of serum albumin redox state, which has long been made exclusively in the context of oxidative stress, has changed dramatically for the past decade.
- Fractions of oxidized albumin elicit a cytokine storm in leucocytes and a respiratory burst of neutrophils, promote the aggregation of platelet and the secretion of a pro-inflammatory cytokine IL-6 in human umbilical endothelial cells, and even facilitate cancer metastasis in cell assays and animal models, all of which are considered to aggravate pathological conditions in various kinds of diseases.
- In animal studies, the redox state of plasma albumin correlates with albumin turnover that is modulated by amino acid/protein nutritional status and is more responsive to protein nutrional status compared with conventional protein nutrition biomarkers, i.e., plasma concentrations of albumin and transthyretin.
- In Japanese pregnant women, maternal serum albumin redox state is associated with infant birthweight, which would be mediated by maternal protein nutritional status as demonstrated by a fetal growth restriction model in rats.
- Clinical studies substantiating the redox state of serum albumin as a protein nutrition biomarker are still insufficient, requring further investigation.

Summary Points

- Serum albumin is the most abundant protein in the circulation and has three isoforms according to the redox state of an cysteine residue at position 34, termed mercaptalbumin, non-mercaptalbumin-1, and non-mercaptalbmin-2, respectively.
- The redox state of serum albumin shifts to a more oxidized state in various pathological conditions such as hepatitis, renal failure, diabetes mellitus, and cardiovascular diseases, as well as physiological conditions such as aging and exercise training. The shift has long been interpreted as the manifestation of systemic oxidative stress accoompanied by these conditions.
- Recent ex vivo studies have shown the possibility that oxidized serum albumin per se could aggravate the pathological conditions of various kinds of diseases.
- A series of recent animal studies have elucidated that the redox state of plasma albumin would be useful as a sensitive protein nutrition biomarker.
- The redox state of serum albumin in Japanese pregnant women has a correlation with their infant birthweight, suggesting its usefulness as an indicator of low birthweight delivery brought about by maternal protein undernutrition.

- The redox state of serum albumin could be also applicable to the assessment of protein undernutrition especially in older population, and clinical studies examining this is warranted as a future direction.

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Branched Short-Chain Fatty Acids as Biological Indicators of Microbiota Health and Links with Anthropometry

4

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Abstract

Bacterial metabolites are one of the main mechanisms of host-microbiota interactions in the gut. Acetate, propionate, and butyrate, also known as short-chain fatty acids (SCFA), are the main final metabolic products of carbohydrate fermentation by the gut microbiota, and both their effects and host health and bacterial production have been substantially studied. However, bacterial metabolites also include the final product of amino acid fermentation isovalerate, isobutyrate, and 2-methylbutyrate, also known as branched short-chain fatty acids (BCFA). Although it is easy to find levels of these metabolites along with SCFA levels in the literature, barely any research in the field of microbiota has given importance to them. In the last few years, coincidentally with the development of high-throughput methodologies for metabolite analysis, some studies have focused in BCFA and related with some health conditions. In this chapter, the relationship between BCFA, diet, and disease will be discussed in order to evaluate them as possible biomarkers of health and/or disease.

Keywords

Isobutyric acid · Isovaleric acid · 2-Methylbutyric acid · Gut microbiota · Branched short-chain fatty acids

Abbreviations

BCAA	Branched-chain amino acids
BCFA	Branched short-chain fatty acids
CE	Capillary electrophoresis
FID	Flame ionization detector
FOS	Fructooligosaccharides
GC	Gas chromatography
GOS	Galactooligosaccharides
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
SCFA	Short-chain fatty acids

Introduction

The human body hosts an estimated number of 500–1000 bacterial species per individual, which accomplish several functions. The recent development of the several -omics technologies gave us principally a descriptive frame of the microbiota in health and disease, including diet. However, researchers are now balancing their efforts with the intention to describe the mechanisms of why and how host-microbiota interactions take place (Gilbert et al. 2018). These questions are important to answer as the biggest question for some of the microbial changes associated with

disease is if they are the cause or conscience of such given condition. One of the main pathways of communication between the host and the gut microbiota occurs through the production of metabolites, especially short-chain fatty acids (SCFA). SCFA are final products of the microbiota fermentation of dietary compounds that reach the colon undigested or partially undigested (Ríos-Covián et al. 2016). Acetic, propionic, and butyric acids are the main SCFA produced by the microbiota and have been extensively studied, but the gut microbiota is also capable of producing low levels of branched short-chain fatty acids (BCFA), which have been scarcely studied in the context of gut-microbiota interactions (Rios-Covian et al. 2020). The main BCFA produced by the microbiota are isovaleric, isobutyric, and 2-methylbutyric acids, which are produced by the fermentation of the branched-chain amino acids (BCAA) leucine, valine, and isoleucine, respectively (Macfarlane et al. 1992). Surprisingly, the main body of research on BCFA is not related with gut microbiota or host health as they are extensively used in material synthesis, food, and pharmaceutical industries (Shi et al. 2019). Additionally, BCFA, specially isovalerate, are believed to be the responsible for the characteristic smell of some food, such as natto or Swiss cheese (Thierry et al. 2004; Hong et al. 2017). As this smell is very strong in the case of natto, there is some research focused in finding ways to reduce the odor of this fermented food through the inhibition of BCFA production route (Hong et al. 2017). BCFA have caught some attention in ruminant research, as protein fermentation occurs in the rumen and the supplementation in steers with BCFA augmented SCFA levels, as well as nutrient digestibility, microbial protein synthesis, and cellulolytic bacteria in the rumen (Liu et al. 2018). The fermentation of BCAA in the colon, apart from resulting in the production of BCFA, entails the production of other metabolites that can be harmful for the colon epithelium such as ammonia, amines, p-cresol, and indole (Mortensen et al. 1992). Humans can also metabolize BCAA to incorporate them in the production of branched medium- and long-chain fatty acids. Recent research suggests that dietary BCFA promote weight maintenance, energy homeostasis, and metabolic health (Taormina et al. 2020). In this book chapter, the process of production of these metabolites by the microbiota, the current methods for its analysis, and the dietary and health factors associated with its fecal levels will be discussed.

Microbial BCFA Biosynthesis

Till date, the synthesis of BCFA by members of the microbiota is not completely deciphered. It is generally assumed that BCFA are produced by the fermentation of the BCAA L-valine, L-leucine, and L-isoleucine, as genes of this route have been described and studied in groups like *Bacillus*, *Escherichia coli*, or *Clostridium acetobutylicum* (Hong et al. 2017) (Fig. 1). In brief, BCAA are converted into a branched-chain 2-keto acid by transamination by the branched-chain α-keto acid dehydrogenase (BCDH) complex. Finally, in the same complex, acyl-CoA and BCFA are liberated. After this, they are available in the lumen for incorporation into the host through the intestinal barrier as it will be explained later in this chapter.

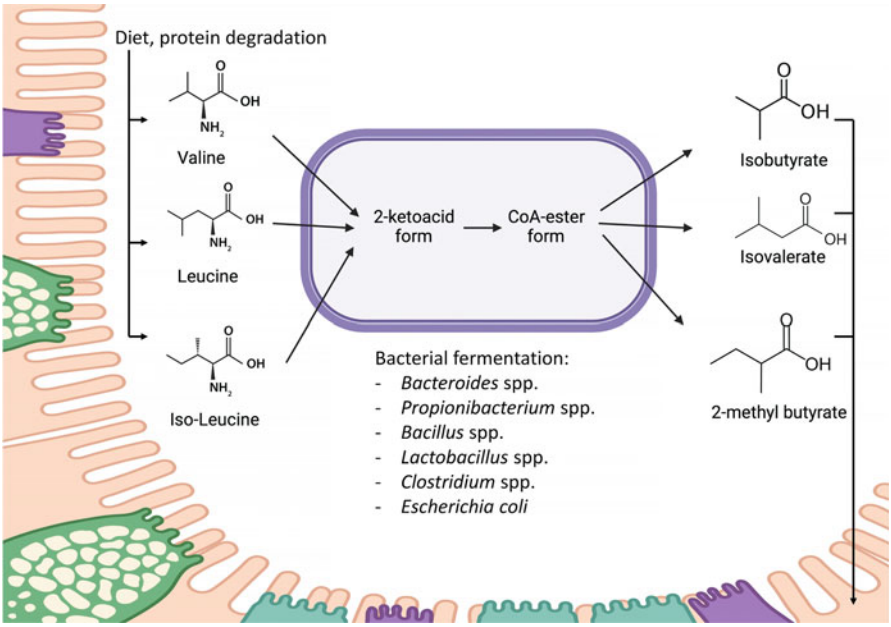

Fig. 1 Fermentation of protein leads to the formation of branched short-chain fatty acids by the gut microbiota. This figure has been done with BioRender

It has been described that in *Bacillus subtilis*, this route produces also the precursor for iso-branched-chain fatty acids $C_{15:0}$ and $C_{17:0}$ through a fatty acid synthase (FAS II), which plays an important role in the fluidity of the cell membrane (Mansilla et al. 2004). Among the members of the gut microbiota, *Bacteroides*, *Propionibacterium*, and *Clostridium* have been described as BCFA producers by several authors (Smith and Macfarlane 1998; Rios-Covian et al. 2015; Aguirre et al. 2016). In addition, some of these studies explore the conditions in which the production of these metabolites is favored by the environment. Smith and Macfarlane demonstrated greater BCFA production when protein-fermenting bacteria were grown in peptides as the main carbon source, but the presence of starch and changes in the pH reduced the production of these metabolites, in particular lower pH values and the presence of fermentable carbohydrates diminished the production levels of BCFA (McFarland et al. 2021). In another study, *Bacteroides fragilis* grown in slowly fermentable carbon sources, where the metabolism is shifted toward protein fermentation, produced more BCFA than when grown in glucose as the main carbon source (Rios-Covian et al. 2015). *Lactobacillus* species used in cheese fermentation also produced isovaleric when grown in vitro with leucine and α-ketoglutarate supplementation, but during cheese fermentation, the production of this BCFA occurs only when *Propionibacterium* species are present (Thierry et al. 2004). In the context of the gut microbiota, it is very complicated to identify which member has the capacity to produce BCFA and what conditions are necessary for it, as it looks that it could be a

metabolic adaptation to protein fermentation when the carbohydrates are scarce as carbon source. This could be the case of cheese fermentation, as *Lactobacillus* could use the lactose of the milk preferably, leaving the protein fermentation to *Propionibacterium*. The fermentation of proteins is one of the main events that occurs in the rumen of ruminants, until now NH_3 was used as an indicative measure of this event, but BCFA might be a better marker for it as it does not serve as a nutrient for other bacteria and they are the final products of bacterial metabolism (Apajalahti et al. 2019). Finally, another possibility that has not been explored in depth is the possibility of a third group of bacteria able to use BCFA as a carbon source or as an structural component, the same way eukaryotic cells and *Bacillus subtilis* use BCFA for the synthesis of longer branched-chain fatty acids.

There are not a lot of studies defining the levels of BCFA produced by the gut microbiota, but from a descriptive point of view, Ríos-Covian et al. reported the differences in BCFA excretion at different time points ranging from 3 months to 95 years of age (with an information gap from 3 months to 18 years). The data obtained highlight the different behavior in fecal excretion of BCFA in infants compared to adults. Contrary to what occurs with total SCFA, which decrease across age, BCFA levels were lower in babies (Rios-Covian et al. 2020). In addition, based on the anthropometric parameters of the adult sample, it was observed that the volunteers classified as extremely obese according to the body mass index (BMI $\geq$ 40) showed higher concentrations of total SCFA and BCFA in faces than the other weight groups (Rios-Covian et al. 2020). Because the diets are uncontrolled in this study, the observed data may be influenced by the underestimation frequently described in obese individuals. Similar results, which will be discussed later in this chapter, were obtained in a recent study recruiting only adults (Cui et al. 2021). Several research focused on SCFA did not analyze BCFA or give no importance to them due to the lack of literature, but as it will be discussed in the next section, BCFA are easy to analyze as they can be determined with the same technologies as SCFA.

Methods for BCFA Analysis

Short-chain fatty acids, and very recently BCFA, in association with health maintenance and disease development resulted in a great interest to develop fast, simple, reproducible, low-cost, and environmental-friendly quantification methods to measure them in different biological samples. BCFA and SCFA are produced in the gut at high concentrations (mM) by microbial fermentation of undigested carbohydrates and proteins and can be transported across the gut and detected in portal, hepatic, and peripherical blood but at lower concentrations (μM) (Ríos-Covián et al. 2016). Thus, the quantification of BCFA in biological samples is a key factor to decipher their potential roles in the human health. Quantification of SCFA in studies has been performed mainly in fecal samples because they are the most accessible biological matrix and are useful enough to study the relationship between SCFA and BCFA production by microbiota in health and disease (Ríos-Covián et al. 2016;

Rios-Covian et al. 2020). BCFA have a different biological origin and functions from SCFA, but their highly homologated chemical structure with that of SCFA means that they can be analyzed together. Several analytical methods have been developed to determine SCFA and BCFA in biological fluids including gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR), and capillary electrophoresis (CE). To increase the sensitivity of SCFA and BCFA detection, several chemical derivatization methods have been reported, and most of the new or modified ones developed for the analysis of gut microbiota-derived metabolites in fecal samples concern SCFA and BCFA (Mojsak et al. 2020).

Gas Chromatography

This method is extensively used due to its compatibility with the chemical properties of BCFA and SCFA, such as volatility, and the suitability of the detectors that can be coupled to this equipment. The flame ionization detector (FID) is commonly used due to lower costs and allows to detect a wide range of concentrations of organic compounds (Ahn et al. 2018). However, mass spectrometry (MS) provides better sensitivity and selectivity compared to FID in complex biological samples (Primec et al. 2017; Hoving et al. 2018). In the case of GC-MS, SCFA and BCFA can be detected directly or by the use of the different derivative agents that converts the analytes into thermally stable, volatile molecules that facilitate the identification in non-targeted analysis (Mojsak et al. 2020). Some authors have described filtration, ultrafiltration, centrifugation, or simple sample dilution techniques to obtain a fast sample preparation in order to avoid the disadvantages of derivatization (Primec et al. 2017). Adding an acidification step to fecal sample extraction has been reported to improve extraction efficiency, and, in addition, SCFA peaks sharpen in chromatograms. Formic acid and phosphoric acid have been the most used acidification agents in fecal extraction (García-Villalba et al. 2012). However, some organic solvents and reagents are harmful to the environment and human health. In addition, the time-consuming pretreatment process increases the reaction time, affects the accuracy and repeatability of the methods, and is one of the reasons for the decrease in detection sensitivity and recovery of SCFA and BCFA, and the development of new methods is an active research area.

Liquid Chromatography

Liquid chromatography equipped with several detectors, such as MS, UV-vis, fluorescence, and electrochemical detector, has been employed in SCFA and BCFA measurement as an alternative method to GC in different types of samples. However, the quantitation of SCFA without chemical derivatization requires harsh experimental conditions due to the strong polarity of SCFA and does not provide good separation on LC columns (van Eijk et al. 2009). In the case of LC-MS, the current goal is to improve the derivatives to improve the retention and ionization

efficiency, resulting in the development of several chemical derivatization methods to quantify SCFA and BCFA (Primec et al. 2017; Mojsak et al. 2020). Isotope techniques employing LC-MS analysis have also developed, improving the sensitivity, specificity, accuracy, and precision. However, some of the derivative reagents were not developed specifically for SCFA analysis, and they have as main disadvantages their potential health and environmental risk, and they require the synthesis of isotope-labeled internal standard compounds, which is difficult and expensive (Han et al. 2015; Mojsak et al. 2020). Finally, LC is a high-cost method due to its maintenance and the requirement of trained professionals and special analytical instruments.

Nuclear Magnetic Resonance and Capillary Electrophoresis

NMR spectroscopy is commonly used for metabolomic studies using cecal content and feces as samples due to its high reproducibility and simple preparation process that normally require minimal pretreatment (Primec et al. 2017).

CE has been mainly used in different biological samples, and the quantification of BCFA is possible in feces (Garcia et al. 2008; Hodek and Křížek 2019). CE separation depends on the electrophoretic mobility of ions through a gel and is dependent on molecular mass and charge of the molecule (Primec et al. 2017). The main advantages are that it can be considered an environmental-friendly technology, it is faster, and it requires minimal pretreatment (Garcia et al. 2008). On the other hand, it presents low repeatability and reproducibility and requires higher metabolite concentration than other techniques.

Branched-Chain Fatty Acids in the Context of Nutrition

Diet is one of the main modifiable factors with a recognized influence on the development of chronic diseases in developed countries. Although several pathways have been described, through which the different dietary components can exert protective actions on the individual's health, the modulating role on the composition and activity of the intestinal microbiota has emerged as a key factor in this association. Since the activity of the microbiota reflected differences between diets, several dietary interventions have been carried out with the aim of modifying the metabolic activity of the microbiota in order to exert beneficial effects on the host health status. Most of them have focused on the administration of different types of carbohydrates such as non-digestible polysaccharides, resistant starch, fructooligosaccharides (FOS), galactooligosaccharides (GOS), or some probiotic strains, with the aim of modifying the production of the major SCFA (acetate, propionate, and butyrate).

An important feature of Westernized societies is the low presence of fiber in the diet (which is often below the recommended daily intakes of 25 g per day) (Jones 2014) in favor of an excessive consumption of proteins from animal origin. In the appropriate amounts, dietary proteins provide nitrogen and essential amino acids for

the host (Trumbo et al. 2002). However, when consumption exceed the daily recommended intake, some proteins escape digestion in the small intestine and reach the colon where they suffer bacterial proteolytic fermentation (Miner-Williams et al. 2012, 2014).

Dietary Interventions Related with Changes in BCFA and Anthropometric Factors

Even though few scientific evidence is available, in a general way, most authors agree in the observation that the administration or the increase of prebiotic carbohydrates into the diets produces an elevation in the SCFA levels along with a reduction of BCFA, whereas the supplementation with amino acids or proteins would result in the opposite effect. While in vitro studies have shown a reduction in the yield of BCFA in vegetarians with respect to omnivores (Wang et al. 2019), recent publications examining this variations in humans have not found differences among them (Trefflich et al. 2021). It is possible, as they suggest, that shifts in the makeup of the bacterial ecosystem between vegetarians and omnivores may drive the functionality of the existing microorganisms in the utilization of the substrates that are available to them, but more evidence is required before to obtain firm conclusions. In addition, in patients with cardiovascular disease undergoing dietary intervention with a vegetarian diet or a meat-enriched diet (based on the Swedish diet with an average consumption of 145 g of meat per day, including red, white, and processed meats) for 4 weeks, no significant differences in BCFA excretion and associated metabolic parameters were found (Djekic et al. 2020).

As mentioned above, one of the characteristics of Westernized diets is the excessive intake of animal proteins. Some authors have focused their efforts on studying the impact of modification or addition of one or more dietary components on fecal BCFA excretion and health status. The replacement of meat proteins in the diet with plant protein-rich foods, which in turn contain a high content of non-digestible polysaccharides and resistant starch, has been shown to be effective in reducing BCFA as well as in the modulation of some blood biomarkers related to lipid profile and glucose metabolism (Venter et al. 1990). Also, supplementation of arabinoxylans in bread exhibited a prebiotic effect, increasing levels of bifidobacteria and lactobacilli and changing the fermentation pattern leading to a decrease reduced combined isobutyrate, isovalerate, valerate, and caproate derived from proteolysis and an increase in butyrate levels, with a known anti-inflammatory effect at the colonic level (Mortensen et al. 1992; Cloetens et al. 2008; Sanchez et al. 2009). An observatory study performed in adult Chinese has also found a positive association between serum butyric and isobutyric acids and body mass index (Wang et al. 2020). Among dietary factors, the intake of insoluble fiber seems to be linked to lower fecal proportions of BCFA (Rios-Covian et al. 2020), whereas dietary fat intake was related with lower levels of valeric, isovaleric, and propionic acids (Cui et al. 2021). Other factors as body mass index, carbohydrate intake, and diverse dietary components showed weak or no correlation with fecal levels of BCFA (Rios-Covian et al. 2020; Cui et al. 2021).

Regardless of the amount of animal protein, evidence from experimental animal studies has demonstrated that the source of protein can influence the bacterial metabolism. Thus, some authors have observed that the levels of total BCFA, isobutyrate, and isovalerate, in the cecum of rats fed red and cured meat, were higher than those of rats fed raw chicken. These differences were also observed at the level of the production of some metabolites such as indole and x-cresol, the fecal content of which was twice as high in animals fed red meat (Van Hecke et al. 2021).

The administration of high-protein diets usually implies a simultaneous reduction in the fiber and vegetable content of the diet, which makes it difficult to draw conclusions. Based on this premise, Mitchell et al. carried out an intervention study in elderly people in which a diet with double the recommended daily protein intake was administered for 10 weeks while controlling the intake of fiber and vegetables. In this case, they did not observe an increase in the proteolytic microbiota or in protein fermentation derivatives, but they did observe an increase in blood concentrations of some metabolites such as TMAO, directly related to the metabolism of some amino acids such as carnitine and choline (Mitchell et al. 2020).

Possible Implications of Branched-Chain Fatty Acids in Host Health

Intestinal proteolytic fermentation mainly occurs in the distal colon, with several factors modulating this process: host gastrointestinal factors, protein-related factors, accompanying dietary components in meals, food processing, macronutrient ratios, and transit time. In general, all conditions favoring a low efficiency of any phase of digestion can increase the availability of proteins for colonic fermentation and hence the amount of BCFA formed, as it is the case of the use of proton pump inhibitors, gastrointestinal surgery, or inflammatory intestinal disease, among others (Zhang et al. 2020; Kiewiet et al. 2021). Besides BCFA, amino acid fermentation gives rise to the production of compounds as ammonia, phenols, amines, hydrogen sulfide, and p-cresol, some of which are harmful for host health (Yao et al. 2016; Oliphant and Allen-Vercoe 2019). Because of this, high rates of colonic proteolytic fermentation accompanied by low levels of saccharolytic fermentation are considered detrimental for host health.

Little is still known about the possible molecular mechanisms of action of BCFA. Preliminary experiments with rat and human adipocytes have shown that isobutyric and isovaleric acids can modulate glucose and lipid metabolism in the liver similar to the major SCFA, then contributing to improve insulin sensitivity in individuals with impaired metabolism (Heimann et al. 2016). In vitro experiments with intestinal cells indicated that isobutyric acid is used as an energy source by colonocytes when butyric acid is scarce (Jaskiewicz et al. 1996) and that isovaleric acid is able to act directly on the colonic smooth muscle causing muscle relaxation (Blakeney et al. 2019). On the other hand, isobutyric acid has been recently identified as a ligand of odorant receptors, the largest subfamily of G protein-coupled receptors, in primary cortical brain astrocytes (Cho et al. 2019).

Isovaleric acid can inhibit Na+, K+-ATP-ase, a key enzyme responsible to maintain the basal potential membrane necessary for a normal neurotransmission (Ribeiro et al. 2009). These last observations provide insights into the participation of BCFA in the gut-brain axis communication.

BCFA in the Healthy Microbiota

BCFA represent a small proportion of total SCFA in feces of healthy humans, usually accounting for less than 5% (Ríos-Covián et al. 2016). Human observational studies indicate that age, sex, and diet could influence the production of BCFA at the intestinal level. Age, in fact, is the factor more strongly and positively associated with fecal BCFA levels (Rios-Covian et al. 2020; Cui et al. 2021); absolute levels of fecal isovaleric as well as butyric, propionic, and valeric acids seem to be higher in men compared to women (Cui et al. 2021). These differences by sex and age could be partly related with differences in dietary patterns as well as with the predominance of proteolytic vs saccharolytic fermentation in the elderly.

Another aspect not sufficiently explored yet is the role that BCFA could play in infant development, and few studies are available establishing associations between factors related with lactation and intestinal BCFA in healthy infants. In vitro fecal cultures and observational studies evidenced higher production of BCFA by the intestinal microbiota of infants fed formula versus those receiving mother's milk, with the addition of 2′-fucosyllactose, lactose, or GOS leading to a decrease in BCFA (Salli et al. 2019; Van den Abbeele et al. 2021). BCFA in infant feces have been positively associated with concentration in breastmilk of phospholipids, free fatty acids, and the level and diversity of oligosaccharides, being the excessive weight gain of infants from 5 to 9 months negatively associated with the content of these compounds in mother's milk (Pekmez et al. 2020).

Changes in the Profile of BCFA Associated with Disease

Several diseases have been related with changes in fecal BCFA, linked in most cases to alterations in the microbiota composition, but only some human studies have considered the study of the SCFA serum profile in certain pathologies, and in most of the cases, the statistically significant results have been mainly associated with major SCFA. In the case of patients submitted to peritoneal dialysis, long dialysis duration, high peritoneal glucose exposure and loss of residual renal function have been associated with alterations in the gut microbiota, as well as, with a reduction of fecal isobutyric and isovaleric concentrations (Jiang et al. 2021). In patients suffering from IgA nephropathy, the levels of the three major SCFA as well as the BCFA isobutyric acid and caproic acid were reduced, which was accompanied by microbiota alterations and a negative association of butyric and isobutyric acids with urea (Chai et al. 2021). The role of BCFA on cardiovascular disease in patients with kidney disease has been also recently explored, and circulating 2-methylbutyric acid was negatively associated

with a certain cardiovascular protein called bone morphogenetic protein 6, suggesting that this pathway may be involved in vascular health in patients undergoing hemodialysis (Wu et al. 2019). Butyric and isobutyric acid levels in stools were significantly reduced in critically ill patients with sepsis as regards healthy people as well as with respect to other diagnostic criteria in hospital intensive care units (Valdés-Duque et al. 2020). The metabolomic profile in non-alcoholic fatty liver disease (NAFLD) patients compared to healthy controls revealed also changes in serum SCFA and BCFA levels being the concentration of butyric, propionic, and isovaleric acids lower in NAFLD subjects (Chashmniam et al. 2019).

In contrast, an increase of BCFA has been evidenced in several pathologies. High levels of fecal BCFA associated with microbiota alterations have been reported in stunted and malnourished children (Li et al. 2019; Surono et al. 2021) as well as in anorexia nervosa (Mack et al. 2016), which is probably related with an increase in colonic protein fermentation due to the scarcity of other fermentable substrates available. Fecal valeric, isobutyric, and isovaleric acids were found higher in colorectal cancer than in healthy individuals (Wang et al. 2017; Niccolai et al. 2019), and it seems to be possible differentiating colorectal and adenomatous polyps patients from healthy controls by the fingerprint of fecal SCFA (Niccolai et al. 2019). Higher abundance of isovaleric and isobutyric acids was found in hypercholesterolemic patients, this last BCFA being also positively correlated with lipid parameters indicative of unfavorable profiles (Granado-Serrano et al. 2019). Intestinal dysbiosis and higher concentrations of fecal propionate and isobutyric acids have been reported in NAFLD (Da Silva et al. 2018), whereas isovaleric acid has been suggested, together with propionic and butyric acids, as possible gut microbiota predictors of obesity in African American men (Barengolts et al. 2019). In a recent study, the relationship between microbiota-derived metabolites, including BCFA, and the degrees of NAFLD was evaluated with women with normal weight or with severe obesity with or without NAFLD. Circulating isobutyric acid levels were significantly lower and isovaleric acid levels significantly higher in women with severe obesity than in women with normal weight (Aragonès et al. 2020).

Rett syndrome is an infrequent X-linked neurodevelopmental disorder that affects female, in which changes in microbiota composition may account for several of the symptoms associated with this pathology. Significantly higher levels of BCFA are associated with Rett syndrome, together with increased microbial genes encoding for propionate, butyrate, and amino acid metabolism (Borghi et al. 2017).

Isovaleric acid is the only BCFA for which a causal relationship has been established between high levels of this compound and human disease. Isovaleric acidemia is a rare inborn error of the metabolism due to a deficiency in the isovaleryl-CoA dehydrogenase, causing the accumulation of isovaleric acid and the disruption of the tricarboxylic acid and urea cycles in the catabolism of leucine. This leads to a metabolic decompensation with encephalopathy accompanied by hyperammonemia and metabolic acidosis in early days of life that, in the case of prolonged or repeated episodes, can result in impaired infant's growth and intellectual disability (Häberle et al. 2018). On the other hand, high stool levels of isovaleric acid have been found correlated in adults with human depression and high cortisol levels, pointing to

an interference of this BCFA with the hypothalamic-pituitary-adrenal axis and/or neurotransmitters (Szczesniak et al. 2016). It is still unclear if BCFA are mechanistically related to these diseases, or they could be used merely as disease biomarkers. In either case, it seems evident that more research is needed to completely understand their role and, probably, use our knowledge about the relationships of dietary compounds and BCFA production to design interventions to improve host health.

Potential Applications to Prognosis and Other Diseases or Conditions

As previously commented during this chapter, it seems to be a link between protein fermentation in the colon, BCFA production, and diet. In addition, changes in BCFA fecal levels have been described in a wide range of pathologies, from cancer to neurological diseases. Thus, these compounds could be used as fecal biomarkers of protein colonic fermentation, as it has been suggested for measuring protein fermentation in the rumen. This fermentation process is considered non-beneficial; therefore, a reduction in the levels of these fecal parameters could be used as a therapeutical target for the reestablishment of a healthier and more saccharolytic microbiota. Nonetheless, in the absence of additional studies, the physiological roles of these BCFA are not currently defined. Despite this interest, there are very few studies in the literature describing BCFA concentrations in humans, so there are currently no mean values that can be used as reference ranges for different population groups according to gender, age, or physiological situation (pregnancy or lactation, e.g.). Since some of these situations result in a negative nitrogen balance and therefore in a lower amount of protein available at the colonic level, this could be of great interest when designing specific recommendations for the modulation of the activity of the intestinal microbiota. In addition, further analyses are needed to understand the extent by which BCFA play a role in the host-microbiota interactions and in the changes in the fecal levels in some diseases, such as anorexia nervosa, malnourishment, colorectal cancer, or Rett syndrome. Whether these compounds are playing an active role or the changes found are coincidental remains unanswered.

Mini-Dictionary of Terms

Microbiota: All the microorganisms that colonize several parts of the host
Short-Chain Fatty Acids: Final products of carbohydrate fermentation by certain groups of bacteria

Key Facts of Gut Microbiota

- Each person can host up to 1000 species, and this microbiota is different from any other individual.

- Gut microbiota accomplish several functions for the host, such as vitamins B and K, development and maintenance of intestine and mucus layer structure, and immunomodulation or protection against pathogens.
- Diet plays a main role in the maintenance of a healthy microbiota. Westernized diet is associated with an unhealthy microbiota.
- Microbiota can be also modulated by probiotic, prebiotic, and synbiotic interventions.
- Other factors like stress, antibiotic, other drugs, pathogens, and age also affect the composition of microbiota negatively.

Summary Points

- BCFA (isovalerate, isobutyrate, and 2-methylbutyrate) are produced by the fermentation of BCAA (leucine, valine, iso-leucine) in the colon.
- Protein fermentation in the colon also produces other non-beneficial compounds, such as p-cresol.
- BCFA can be analyzed with the same methods used for the analysis of SCFA.
- BCFA are associated with an increase of protein and a reduction of complex carbohydrate intake.
- BCFA fecal levels increase with age and are higher in males than in females.
- Changes in BCFA have been found in several health conditions, such as anorexia nervosa, malnourishment, colorectal cancer, or Rett syndrome.

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Plasma Nitrate and Nitrite as Biological Indicators of Health and Disease in Nutritional Studies

5

Keith R. Martin and Richard J. Bloomer

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Abstract

Dietary vegetables represent a nitrate-rich source for humans, and consumption increases plasma levels of nitrate two- to threefold. Both dietary nitrates and nitrites can also be bioactivated endogenously to form NO and other nitrated bioactive molecules. The relative critical importance of NO and its vasodilatory function, among many others, have garnered considerable research interest. Research efforts to monitor NO formation and correlate with potential beneficial health outcomes are hindered by the extremely fast half-life of NO. However, the immediate metabolic byproducts of NO formation are nitrate and nitrite. Given the considerable stability of the former, it has been used routinely and effectively in nutritional studies as a surrogate biomarker, although indirect, for NO production. From these studies, dietary nitrates and purportedly NO have been correlated with myriad beneficial health outcomes in nutritional studies. Further investigation is needed regarding physiological functions and therapeutic applications of dietary nitrates with a focus on large epidemiological studies.

Keywords

Nitrate · Nitrite · Nitric oxide · Biomarker · Nutritional studies · Laboratory assessment · Griess method · NO metabolites · NO half-life · NOx

Abbreviations

ADI	Acceptable daily intake
ATP	Adenosine triphosphate
CHF	Congestive heart failure
DBP	Diastolic blood pressure
eNOS	Endothelial NOS
EPR	Electron paramagnetic resonance
FDA	Food and Drug Administration
FMD	Flow-mediated dilation
GC-MS	Gas chromatography-mass spectrometry
ICAM-1	Intercellular adhesion molecule, isoform 1
iNOS	Inducible NOS
LC	Liquid chromatography
NADPH	Nicotinamide adenine dinucleotide
nNOS	Neuronal NOS
NO	Nitric oxide
NOCs	N-nitroso compounds
NOS	Nitric oxide synthase

NOx	Nitrate and nitrite
RSE	Red spinach extract
SBP	Systolic blood pressure
USDA	United States Department of Agriculture
VCAM-1	Vascular cell adhesion molecule, isoform 1

Introduction

Definition of Nitrates

Nitrates and nitrites are classes of compounds containing nitrogen and oxygen, which can exert biological activities. From a nutritional perspective, they are thought to be prevalent primarily in cured, dietary meats. However, the concentrations are considerably greater in green leafy vegetables. Dietary nitrates occur also in water sources, other fruits, and vegetables and as purified salts and dietary supplements. Upon consumption of nitrate-rich dietary sources, plasma nitrate increases around two- to threefold and remains elevated for up to 2 weeks from a bolus dose. The capacity for nitrates and nitrites to form nitric oxide (NO) is of considerable physiological relevance. NO has been routinely used as a therapeutic agent particularly for mitigation of myocardial ischemia for more than 130 years without direct knowledge of its role in vasodilation. The formation and/or release of NO from precursor molecules, e.g., oral organic nitrates, produces inorganic anions nitrite $\left(NO_2^-\right)$ and nitrate $\left(NO_3^-\right)$, which have previously been considered inert end products of NO metabolism and/or residues in the food chain. There has been significant recent interest in the use of nitrate and nitrite as biomarkers of NO formation and metabolism.

Naturally occurring nitrate and nitrite are physiologically recycled in blood and tissue to form NO as well as many diverse bioactive nitrogen oxides. As a result, nitrates purportedly are storage mechanisms for NO-like bioactivity as an adjunct to the endogenous enzymatic pathways. The two major physiological sources of NO are the endogenous, enzymatic NO synthase (NOS [iNOS, eNOS, nNOS]) pathways requiring L-arginine and oxygen as substrates and occurring in three isoforms as inducible, endothelial, and neuronal (Fig. 1). The exogenous consumption of dietary nitrates occurs primarily via vegetables, water, and dietary supplements. For each, formation of NO requires bioactivation of nitrate via chemical reduction to nitrite, which is largely due to >300 species of commensal bacteria in the oral cavity. After extraction, conversion, and concentration, salivary nitrates are swallowed (~1.5 l/day), converted to nitrite in the gastric lumen, and distributed systemically. Around 80% is filtered out in the kidneys and 20–25% reabsorbed back into circulation, combined with other nitrogenous compounds produced endogenously and the cycle repeated as the enterosalivary pathway (Fig. 1). The numerous other endogenous molecules (both enzymatic and non-enzymatic) that can produce NO include hemoglobin, myoglobin, xanthine oxidoreductase, ascorbate, and polyphenols.

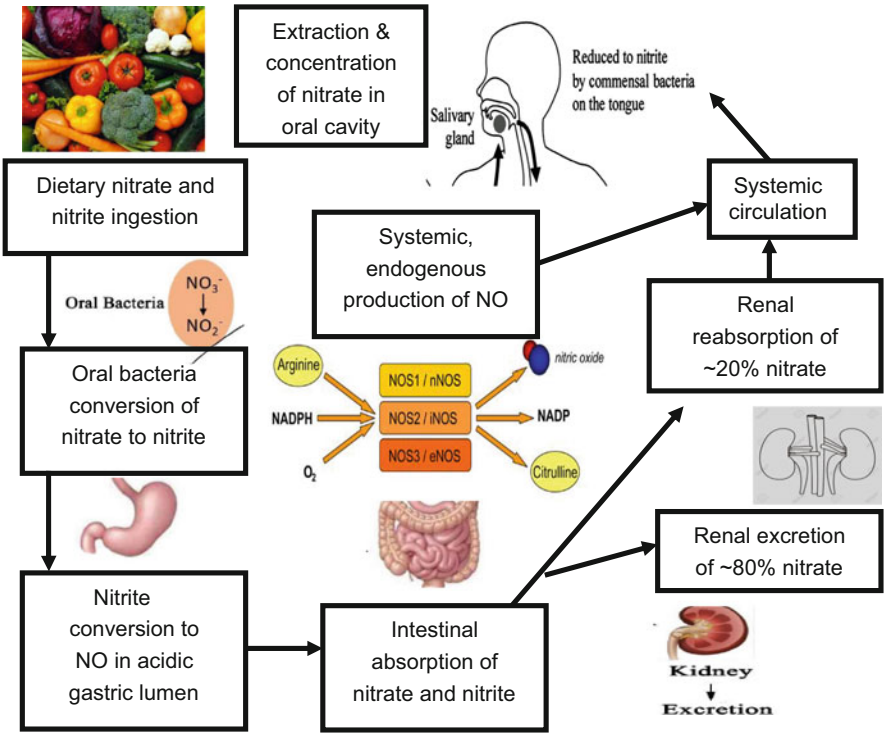

Fig. 1 Dietary nitrate and nitrite ingestion and circulation in the body. Dietary nitrates are consumed via nitrate-rich plants and water, which increases plasma levels two- to threefold. Nitrates can then be reduced to other bioactive nitrogen oxides in the gastric lumen and systemically to produce the potent vasodilatory nitric oxide. Approximately 20–25% of nitrate is reabsorbed and combined with endogenously produced nitrates in circulation where it is extracted by the salivary glands and converted and concentrated to other nitrogenous compounds in the enterosalivary pathway

Sources of Dietary Nitrates in Nutritional Studies

Foods Containing Nitrate

Myriad vegetables such as lettuce, spinach, and beetroot accumulate significant amounts of nitrate (~80% of total dietary) from nitrogen-based fertilizers. Other examples include radishes, turnips, watercress, bok choy, Chinese cabbage, kohlrabi, chicory leaf, celery, onion, and garlic (Kalaycıoğlu 2019). Fruits such as watermelon, apples, bananas, grapes, kiwi, pears, oranges, and strawberries also contain nitrates but at low levels (Salehzadeh et al. 2020). Current estimates of dietary nitrate consumption in the USA range from 40 to 100 mg/d.

Nitrate as a Food Additive in Processed Meats

Nitrates are routinely added to processed, cured meats as antioxidants, flavor enhancers, color stabilizers, and antimicrobial agents. Moreover, nitrate inclusion is critical for minimizing or preventing the growth of disease-causing bacteria such as *Clostridium botulinum*, the causative agent of botulism (Dahle 1979). Nitrate-enhanced foods include bacon, bologna, corned beef, hot dogs, ham, luncheon meats, sausages, canned meat, cured meat, and hams (Lee 2018). Nitrate and nitrite concentrations of foods are regulated by the US FDA and USDA, and according to the Code of Federal Regulations, sodium nitrite concentrations in cured meats must be $\leq$200 ppm (mg/kg), and sodium nitrate must be $\leq$500 ppm (mg/kg). The addition of nitrates and nitrites to foods, i.e., cured meats, has generated concern among many due to the association with nitrosamine formation in the acidic gastric lumen and occurrence of gastric cancer. However, food additives are not major contributors to the total estimated dietary intake of nitrates.

Nitrate as a Dietary Supplement

Inorganic nitrate and nitrite are plant nutrients and legally permitted food additives primarily for processed meats and components of foods. They also may be included in dietary supplements and nutraceuticals since they are associated with blood pressure-lowering and athletic performance-enhancing effects. As a result, supplementation is very popular among consumers with cardiovascular disorders as well as competitive athletes. The most commonly used dietary constituent is nitrate-rich beetroot because of its high nitrate concentration (>250 mg nitrate/100 g [3.5 oz]) coupled with compelling evidence that it does, in fact, increase blood nitrate and/or nitrite concentrations following acute and chronic ingestion. Others have shown that dietary nitrate supplementation with ~0.1 mmol/kg body mass significantly reduces blood pressure, reduces oxygen cost associated with exercise, improves muscle efficiency, and exerts ergogenic properties (Jones 2014). In a comparison of dietary supplements, i.e., nitrates, there was considerable evidence supporting the exertion by nitrates of acute beneficial effects on muscle strength (Valenzuela et al. 2019). As previously discussed, the mode of action is purportedly the facilitated NO bioavailability and subsequent vasodilation leading to improved cardiovascular health and enhanced endurance, tolerance, and athletic performance (Clements and Lee 2014).

Given the growing popularity of nitrate-rich supplements, there are many in development or already on the market that have shown efficacy. For example, the blend of some plant-based ingredients (beetroot, red spinach (*Amaranthus tricolor*), and aronia berry extracts) yielded a significant increase in plasma NO metabolites following acute ingestion (Bloomer et al. 2020). Moreover, consuming only 4–12 mg/kg of nitrate (300–800 mg/d) as a dietary supplement, e.g., beetroot juice, beetroot concentrate powders, and/or sodium nitrate, conferred significant

cardioprotection (Jackson et al. 2018). Reductions in blood pressure and platelet aggregation at similar dietary amounts with 4–20 mg/kg have also been observed (Bryan and van Grinsven 2013). Most nutritional studies have used nitrate-rich beetroot as a dietary supplement although many dietary supplements and nutraceuticals are available commercially.

An evolving paradox regarding dietary nitrate supplementation focuses on whether benefit outweighs detriment when intake may exceed recommendations for daily intake. For example, consumption of a single serving of a nitrate-rich food or dietary supplement can exceed the acceptable daily intake (ADI) for nitrate (222 mg/d for a 60 kg adult) per the World Health Organization (Mensinga 2003). Recommendations by experts regarding dietary supplement intake in exercise and athletic performance are 300–600 mg of nitrate (<10 mg/kg or 0.1 mmol/kg) or 500 mL beetroot juice (3–6 whole beets) within 1.5 h of exercise commencement (Vitale and Getzin 2019). Others advise multi-day dosing for a week prior to exercise or an athletic event. Given the potential health benefits and risks for dietary nitrate and nitrite intakes, evidence-based dietary recommendations regarding nitrate- and nitrite-containing foods and dietary supplements would be welcomed to facilitate optimal cardiovascular health and athletic performance without risk of adverse events.

Nitrate and Nitrite Salts

Supplementing directly with inorganic sodium or potassium nitrate has been considered by many athletes and/or infirmed individuals, e.g., cardiovascular disease. Both salts are available commercially; however, their indiscriminate use and assumption of safety without corroborating evidence have generated considerable concern. Health professionals are particularly concerned about nitrite, which has an LD50 (lethal dose to 50%) similar to cyanide and can readily cause death (Lundberg et al. 2011). The LD50 for nitrite is 100–200 mg/kg body weight, which is 7–14 g (0.25–0.5 ounces) for a 70 kg (154 pound) human (Lundberg et al. 2011).

Beets and Beetroot Juice

The most commonly used dietary constituent thought to enhance plasma NO levels is beetroot in some form or its juice because of its high nitrate concentration coupled with reports that dietary consumption increases plasma nitrate and/or nitrite subsequent to both acute and chronic ingestion (Kapil et al. 2015). The results, however, have been inconsistent and likely due to the large range of concentrations of nitrates in vegetables and their juices. This can easily occur due to agricultural and agronomic practices, e.g., nitrate-rich fertilizer use (Gallardo 2019). The overwhelming consensus, however, is that nitrate-rich beetroot juice is efficacious in several aspects of cardiovascular health as well as physical performance and thus a good selection for a nitrate-rich dietary source, which also permits comparison of study results via meta-analyses (McDonagh et al. 2019). Other diverse products and/or blends, such vitamins, fruits, synthetic products, natural products containing nitrite, or spices, which have similar properties of nitrites, are also undergoing experimental testing (Gassara et al. 2015).

Beetroot juice is gaining increasing attention in the context of athletics and exercise performance due to emerging evidence for its role in vasodilation and subsequent reductions in blood pressure and increases in oxygenation (reduced ischemia) and nutriture of various tissues particularly exercising muscle that may become hypoxic. Dietary nitrates can also increase muscle efficiency and exercise tolerance and markedly improve endurance (Zamani et al. 2021). Other enhanced functions include increased blood flow (hemodynamics), improved gas exchange, enhanced mitochondrial biogenesis and efficiency, and strengthening of muscle contraction (Domínguez et al. 2018). In a meta-analysis of 22 studies, beetroot supplementation significantly increased cardiorespiratory endurance, exercise efficiency, athletic performance, time to exhaustion at submaximal intensities, and cardiorespiratory performance at intensities approaching anaerobic threshold and VO_{2max} (Domínguez et al. 2018).

Support for evidence-based sports performance supplements (caffeine, creatine, nitrate/beetroot juice, β-alanine, and bicarbonate) depends on the nature of the athletic event, the inherent attributes of the event, and the individual responsiveness to the dietary supplement or nutraceutical. These assertions corroborate that effects are dependent on dose and duration of exercise and in particular time to exhaustion seems to increase and ergogenic benefits may depend on individual aerobic fitness level (Rothschild 2020). Individuals with a lower fitness level may benefit more in athletic or exercise performance after dietary nitrate consumption than better-conditioned (higher fitness) athletes presumably via improved oxygen cost and consumption during exercise with greater energy production, viz., ATP, and lower energy consumption. Collectively however, a meta-analysis of beetroot juice studies revealed considerable, beneficial effects on athletic and exercise performance when athletes, non-athletes, and modes of exercise were compared (Braakhuis 2015). Dietary nitrate supplementation also exerts notable effects on training performance in patients with peripheral artery disease, heart failure, and chronic pulmonary obstructive disease. Although promising, larger randomized controlled trials are needed to corroborate and confirm the overall efficacy of beetroot as a dietary supplement (Peeling et al. 2018).

Red Spinach

Red spinach extract (RSE) is a second well-known nitrate-rich dietary supplement. RSE consumption significantly increases plasma nitrate 30 min post-ingestion, with significant microvascular reactivity in the vascular resistance vessels with consequent improvement in hemodynamics of the lower limbs of humans (Haun et al. 2016). Dietary intake of RSE (1000 mg dose; ~90 mg nitrate) significantly increased plasma concentrations of nitrate in both pre- and post-graded exercise testing GXT, but not with placebo. VO_2 at the ventilatory threshold was significantly higher after RSE ingestion compared to placebo suggesting that ergogenic properties are improved via modulating the ventilatory threshold (Moore et al. 2017). In a study of 17 recreationally active men and women, participants were supplemented daily with 1 g of RSE or placebo for 7 days prior and 1 h before completing 2 randomized testing sessions of a 4 km cycling time trial test (Gonzalez et al. 2019).

RSE supplementation significantly reduced post-exercise diastolic blood pressure and increased 4 km completion time, average power, relative power, and average speed. Only females displayed significant improvement during RSE trials (Wickham and Spriet 2019). RSE supplementation also significantly reduced time to completion, increased measures of power and speed, and lowered post-exercise diastolic blood pressure during a 4 km cycling time trial without altering subjects' perceived exertion or subjective measures of muscle fatigue.

Nitrates and Nitrites in Nutritional Studies

NO is a highly reactive, labile, gaseous signaling molecule and free radical. It is involved in a variety of physiological and biochemical processes; thus, its metabolites are routinely measured to determine indirectly NO levels and correlated with beneficial health outcomes. Although it has been associated with virtually every organ system in the body, its most important roles are regulation of blood flow, i.e., hemodynamics, in the vascular endothelium (vascular homeostasis), neurotransmission, and host defense mechanisms.

Half-Life of Nitrate and NO

Once generated and bioactive, NO can exist as the nitronium cation (NO_2+), the nitroxyl anion (NO-), and a NO free radical with half-lives <20 s in peripheral circulation. Subsequently, these entities proceed via sequential oxidation or nitrosation of water to form NO metabolites with greater stabilities such as nitrite [$t_{1/2}$ = 110 s in blood] and nitrate [$t_{1/2}$ = 5–8 h in blood]. Nitrate and nitrite are collectively referred to as NOx and can be recycled to produce NO (via reduction) in myriad reactions or processes including the enterosalivary circulation of endogenous nitrate, an essential pathway in the maintenance of NO homeostasis. NOx in blood has routinely been used as an index of eNOS activity and as an indirect measure of NO levels. However, differences in dietary intake of these chemical moieties can vary considerably and shift production sources, which may confound some study results. For example, during fasting conditions with low dietary intake of NOx, enzymatic formation of NO via NOS accounts for the majority of circulating NOx.

The quantification of NO metabolites in biological samples provides meaningful information concerning endogenous NO production, bioavailability, and metabolism. However, due to the limited physiological half-life of NO, different approaches for the detection of the NO reaction products have been under development. The major pathway for NO metabolism is the sequential oxidation to nitrite and nitrate rendering both useful as biomarkers (Yoshida et al. 1983). In plasma and other physiological fluids and compartments, NO is oxidized primarily to nitrate, where it remains stable for several hours (Kelm et al. 1992). The half-life of nitrite in human blood is ~2 min, whereas nitrate, on the other hand, has a circulating half-life of 5–8 h (Tannenbaum 1994).

Nitrate and Nitrite as a Biomarker

As a result of low dietary intake of nitrite/nitrate, enzymatic NO formation from NOS synthesizes the majority of physiological nitrite (Rhodes et al. 1995). An initial assumption was that NO is oxidized to nitrite and nitrate, but studies indicate that nitrite or nitrate can be recycled to produce NO via myriad means (Fig. 2). Thus, accurate detection of both anions (nitrate and nitrite) becomes crucial in NO biology. There have been assumptions that nitrite concentrations would correlate with real-time NO status better than nitrate because nitrite is the immediate, first decomposition product of NO, nitrite is not frequently present (to any great extent) in food or water, and plasma nitrite and nitrate could be significantly altered when moderate changes occur with NO production. Moreover, the disadvantages of nitrate as a NO marker are that high plasma nitrate concentrations render an immediate change in the NO concentrations, which may be difficult to detect. Since dietary nitrates are prevalent, restricted dietary intake may cause a physiological decrease in nitrate concentrations without a consequent decrease in NO production. Urinary elimination via NO-induced vasodilation is also a consideration.

In animal studies, plasma concentrations of nitrate considerably fluctuate following administration, which were both qualitative and quantitative. This suggested that

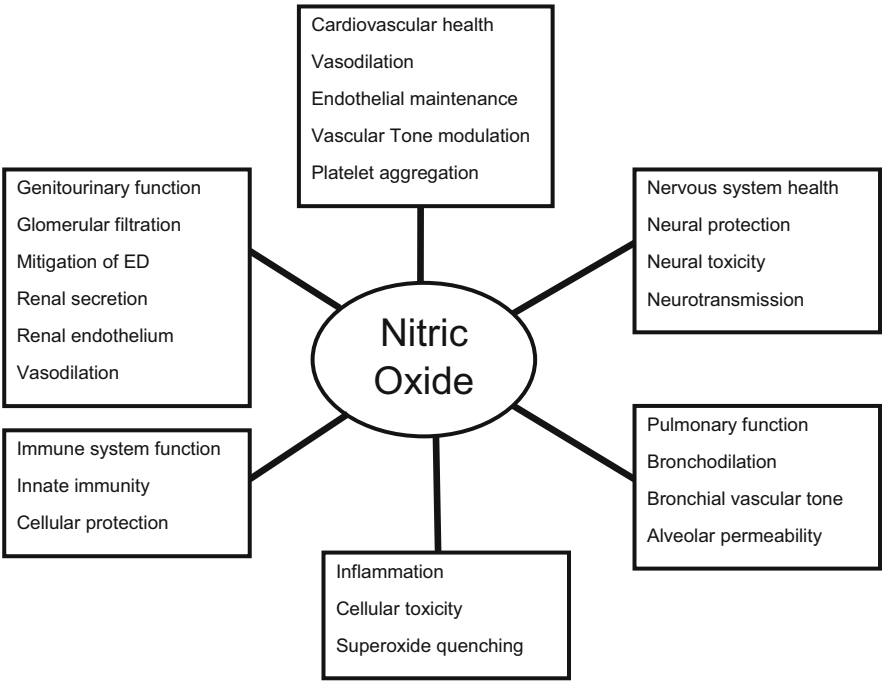

Fig. 2 The myriad functions and interactions of exogenous and endogenous nitrates and nitrites on biological function. Illustration of the myriad, diverse effects of NO on different physiological functions in the body

changes in plasma nitrite concentrations are more predictable than those of nitrate. As a result, NOx (nitrate and nitrite) concentrations measured as a sum of nitrite and nitrate, as it is done frequently in scientific literature for the assessment of human NO status, could lead to misinterpretation of data. Thus, nitrate concentrations alone are not always a reliable predictor of the NO status unless relative changes in plasma nitrite are also considered. Unfortunately, many studies published in the scientific literature do not quantitate nitrite potentially limiting the veracity of the outcome.

Importance of NO Concentration

Nitrate and nitrite have been shown to be reliable biomarkers for cardiovascular diseases as well as other chronic diseases from both diagnostic and therapeutic perspectives. In addition to blood (serum and plasma), urinary levels of NOx provide a means to assess systemic production of NO in vivo with the understanding that urinary (or plasma) levels are truly reflective of endogenous production of NO and dietary ingestion of NOx as well as from bacterial activity in the gut (Lundberg et al. 2008). Other biological sources of nitrate often measured in studies include nasal washes, cerebrospinal fluid (CSF), and the fractional exhaled NO from the lungs (Fig. 3). The interaction of NO

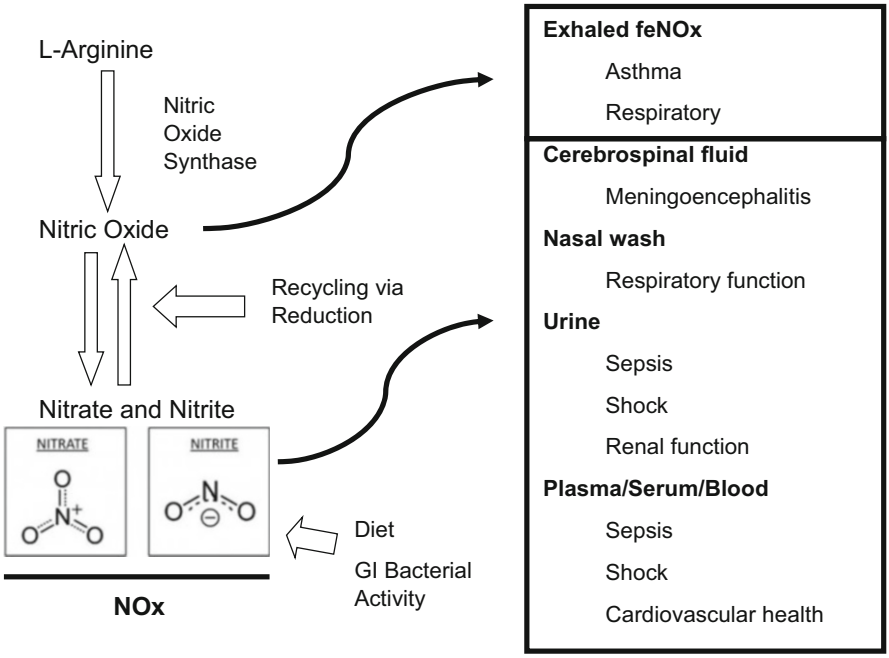

Fig. 3 Interaction of nitric oxide (NO), nitrite, and nitrate. Generation of NO via oxidation to nitrate or nitrite (notated as NOx) and detection as biomarkers of health and disease. feNOx: fractional excretion of nitric oxide. (Adapted from Mian, A. et al. The Open Biochemistry Journal, 7(1):24–32, 2013)

and NOx with other chemical moieties such as superoxide (producing peroxynitrite), thiols (producing S-nitrosothiols), secondary amines (producing N-nitrosamines), and metals (producing nitrosyl-heme) is also important physiologically and potentially useful from the perspective of biomarkers. As a free radical, NO is bactericidal although it may scavenge superoxide to protect against injury. This seemingly paradoxical reaction occurs via peroxynitrite, a potent cytotoxic agent, implicated in disease processes. As a result, it is an area of intense research effort.

Indirect Physiological Biomarkers of NO Exposure

Numerous studies have evaluated beneficial effects related to the intake of nitrate or nitrite, by monitoring plasma levels and correlating with indirect physiological markers such as mean diastolic and/or diastolic blood pressure as a surrogate short-term biomarker predictive for long-term beneficial effects. In addition to blood pressure, further biomarkers related to CVD risk, such as those arising from alterations of circulatory parameters and blood flow, i.e., hemodynamics, platelet adhesion and/or aggregation, and maintenance of vascular tone. There is a need to adequately characterize the potential health risk associated with enhanced dietary nitrate intake and to develop biomarkers indicative of both endogenous and exogenous N-nitroso compounds (NOC) exposure. For example, the urinary excretion of non-carcinogenic N-nitroso-amino acids may be used as a readily accessible biomarker for endogenous N-nitrosation. To corroborate the correlation of such biomarkers with the risk resulting from the in vivo generation of carcinogenic NOCs or corresponding genotoxic intermediates, specific DNA adducts as indicators of genotoxic damage by NOCs in human blood leukocytes or biopsy samples can be used. Validation would require corroboration using human blood and/or tissue samples to assess usefulness as predictive biomarkers particularly via the execution of prospective epidemiology studies.

NO and its Metabolites in Diseases and Pathological States

In addition to the various physiological and biochemical properties and functions described above, NO can also be used a biomarker for many inflammatory disease states, either local to the tissue where it is generated or systemically. The effects of NO as an inflammatory mediator have been studied in detail. NOS and NO induction have been shown to be innate immune effectors of infectious/inflammatory pathways. A recent study showing the temporal effects of NO metabolites in a rodent model of systemic inflammation and sepsis revealed tissue-specific and time-specific changes in NO metabolites. Interestingly, NO seems to be a mediator in inflammatory disease but also a cytotoxic effector of non-specific immune response. Moderate levels of NO are beneficial, whereas excess levels are detrimental and cause damage (Mian and Aranke 2013).

Both direct and indirect detection techniques of NO have been developed and are widely used. Direct detection methods include chemiluminescence, electron paramagnetic resonance (EPR), and the use of electrochemical sensors, which can detect NO in various biological environments although some experimental designs may preclude their use due to cost, feasibility, and confounding of the design, e.g., facilitation of real-time sample collection. Indirect methods typically measure redox-related species of NO (nitrite or nitrate) or products formed during the interaction of NO-derived species with biological molecules such as protein, carbohydrate, lipid, thiols, etc. Overwhelmingly, the most frequently used technique for the estimation of nitric oxide status, in both animal and human experiments, is the determination of nitrite and nitrate by the Griess method as the total amount, viz., NOx. This has, however, come under scrutiny due to the different sources of production and the chemical interconversions as mentioned previously.

Laboratory Assessment of Nitrate and Nitrite

The Griess assay is one of the most commonly used assays in nutritional studies to quantitate nitrate and nitrite (Wishnok and Glogowski 1996). The main reagents used for this assay are enzymatic nitrate reductase, lyophilized reduced β-nicotinamide adenine dinucleotide (NADH), and Griess reagents, which include sulfanilamide and N-(1-naphthyl)ethylenediamine in hydrochloric acid. Total nitrite concentrations (NOx) are determined by enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product, which is based on a two-step diazotization reaction. Acidified nitrite produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl)ethylenediamine to form the chromophoric azo-derivative, which absorbs light at 540–570 nm. The total concentrations of nitrite and nitrate are calculated using a standard curve constructed using purified sodium nitrite. It is noteworthy that nitrite is labile and can be quickly oxidized to nitrate after blood collection unless analysis is expedient. Additionally, using colorimetric detection, the total nitrite and nitrate levels are in the μM range, whereas the nitrite level is in nM range. Overall, however, there are fairly low laboratory variability, good stability with delayed processing, and modest within-person reproducibility over time with this assay supporting its use as a biomarker in epidemiologic studies.

The Griess assay combined with colorimetric detection is simple and easy to use compared to gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC) detection. However, this assay is not sensitive enough to detect nitrite. Measuring circulating levels of nitrate is valuable not only for evaluating nitrate exposure and risk of chronic disease but also for monitoring changes of nitrate in nutritional nitrate intervention studies since plasma levels of nitrate can be reliably measured and employed in large epidemiologic studies with acceptable reproducibility and marginal risk of laboratory error (Wang et al. 2013).

Nitrate as a Biomarker to Determine Potential Health Benefits of Nitrate

Cumulative evidence clearly suggests that nitrates may exert numerous health benefits. Nitrates can produce NO, which readily reduces blood pressure as an indirect measurable biomarker via its vasodilatory effects. Subsequently, the risk for cardiovascular disease, coronary heart disease, myocardial infarction, and stroke are markedly reduced after dietary nitrate consumption (Siervo et al. 2018). In a meta-analysis of 34 studies, inorganic (pharmacological) nitrate consumption significantly reduced blood pressure, improved endothelial function, reduced arterial stiffness, and reduced platelet aggregation (Woessner et al. 2018). Other beneficial effects include improvement and/or reduced risk for gastric ulcers, renal failure, and metabolic syndrome as well as neural function and immune function (Raubenheimer et al. 2019).

Increased NO

Numerous studies have reported significantly increased plasma nitrates after ingestion of nitrate-rich foods and dietary supplements. Ingestion of high-dose nitrate as either synthetic sodium nitrate or natural beetroot juice in eight young healthy individuals rapidly increased plasma nitrate concentration up to threefold, which was maintained for 2 weeks (Miller et al. 2012). Mayra et al. showed in a crossover trial that those consuming high-nitrate green leafy salad twice daily for 10 days significantly increased fasting plasma nitrate/nitrite concentrations determined by the Griess method and significantly improved vascular dilation or relaxation using flow-mediated dilation (FMD) by 17% (Mayra and Johnston 2019). In hypertensive pregnant females, daily nitrate (70 mL for 8 days) consumption significantly increased plasma and salivary nitrate/nitrite concentrations compared to placebo and was significantly correlated with corresponding reductions of diastolic BP (Ormesher et al. 2018). For studies demonstrating protective effects after nitrate consumption but without measuring plasma nitrate/nitrite levels, there is an assumption that levels did increase to elicit the effect provided the study was placebo-controlled, well-designed with fairly stringent inclusion/exclusion criteria.

Improved Blood Flow: Response to Hypoxia

Dietary and medicinal nitrates such as organic nitroglycerin have been pivotal therapeutic and pharmacological agents for the mitigation of cardiovascular disease for decades. Nitrates cause potent vasodilatation of the capacitance veins and substantially improve cardiac ventricular filling pressure and dilate the inner layer of the pericardium coronary arteries, improving blood flow, particularly in ischemic (hypoxic) tissue (Goodwill et al. 2017). Bioactivation of organic (and inorganic) nitrates generates NO, which induces potent vasorelaxation via its effect on vascular

smooth muscle cells embedded in the vasculature. Platelet activation and subsequent aggregation, or clumping, are also inhibited reducing the risk for thromboemboli.

Exercise can induce hypoxia and reduce muscle oxidative function impairing the capacity to sustain aerobic exercise. Subjects consuming nitrate-rich beetroot juice (750 ml) displayed sustained limits of tolerance during hypoxia, whereas the placebo group displayed impaired levels. There was also substantial reduction of skeletal metabolic perturbation during hypoxic exercise. Beetroot ingestion also improved muscle energetics and functional capacity during hypoxia. Beetroot juice concentrate provided to healthy subjects caused marginal but statistically significant improvements in arterial oxygen saturation between placebo and nitrate after 2 min of static apnea (Engan et al. 2012). Maximal apneic duration was increased by 11%.

Consumption of 250 mL beetroot juice compared to water prevents endothelial dysfunction caused by hypoxic ischemia reperfusion as well as reduces arterial stiffness (Kapil et al. 2010). In non-medicated hypertensive adults, beetroot juice (500 mL for 15 days) significantly reduced proatherogenic adhesion molecule expression (ICAM-1, VCAM-1, E-selectin) (Asgary et al. 2016). In patients with hypercholesterolemia, consumption of 250 mL beetroot juice per day significantly induced vasorelaxation with increased vascular diameter (Velmurugan et al. 2016). Other studies have corroborated these observations demonstrating that acute ingestion of beetroot juice (2–3 h with 341–1488 mg) significantly mitigated ischemic reperfusion injury (Webb et al. 2008).

Reduction in Blood Pressure

Numerous clinical studies have reported the capacity for nitrate-rich beetroot juice or some other NO donor to reduce blood pressure (Siervo et al. 2013). For example, sodium nitrate (8.5 mg/kg/d) consumed for 3 days increased plasma levels of nitrate and significantly reduced DBP by 4 mmHg in young healthy volunteers. A bolus dose of beetroot (500 ml; 1,400 mg) reduced both SBP and DBP by 10 and 8 mmHg, respectively. Subsequent studies using beetroot juice (250 ml) produced a 5 mmHg reduction but only in SBP. Several studies have now confirmed the blood pressure-lowering activity of dietary nitrate mostly in healthy young adults although similar effects have been observed in older adults (Oggioni et al. 2018). A single bolus dose of beetroot juice to older adults with peripheral artery disease significantly reduced blood pressure, increased time of claudication pain, and prolonged walking time. Dietary nitrate supplementation reduced muscle fatigue caused by exercise-induced damage to contractile muscle. Dietary nitrates also reduced the perception of effort and leg muscle pain during exercise (Husmann et al. 2019). Different doses of beetroot juice (100, 250, 500 mL) consumed over 24 h increased plasma levels and reduced ambulatory blood pressure in healthy adults and were dose-dependent with reduced systolic, but not diastolic, blood pressure (Hobbs et al. 2013).

Numerous studies have reported the anti-hypertensive action of dietary nitrates and nitrate-rich vegetables in healthy non-smokers; daily consumption of 70 mL of

beetroot juice significantly reduced both mean arterial and diastolic blood pressures (Keen et al. 2015). In two clinical trials, significant reductions in systolic and diastolic blood pressure were noted in a dose-dependent (100–583 g) manner with increased endothelium-independent vasodilation (Hobbs et al. 2012, 2013). In other studies, oral inorganic nitrate and nitrite infusion significantly dilated peripheral arteries leading to increases in forearm blood flow. In clinical studies of hypertensive patients, dietary inorganic nitrate (beetroot juice) produced sustained blood pressure drops. Compelling evidence demonstrates that dietary consumption of synthetic dietary nitrates or nitrate-rich vegetables significantly increases plasma nitrate and decreases blood pressure within hours of ingestion and is correlated with dose.

General Cardioprotection

Cardioprotection includes all mechanisms that collectively protect the heart with subsequent inhibition of myocardial injury. Results of studies indicate that nitrate ingestion reduces infarct area and improves clinical outcomes, although the mechanisms are unclear. Nitrates can modulate events in myocardial infarction, an ischemic, hypoxic event, through several mechanisms (Baker et al. 2007). Nitrates can improve hemodynamic (blood flow) effects and increase collateral flow of the adjunct microvasculature as well as facilitate and temper reperfusion or prevent adverse remodeling changes including changes in size, mass, and function of the heart. Coronary blood flow may be redirected to tissue with low oxygenation. In a study of coronary heart disease (CHD), the highest quintile of nitrate consumption, compared to the lowest, there was a protective association for CHD (relative risk 0.77).

In patients with congestive heart failure (CHF), peripheral abnormalities are apparent, including a high degree of vasoconstriction relative to a maximally dilated state and ultrastructural changes to cellular architecture of skeletal muscle. Recent studies in patients with CHF with preserved ejection fraction (% blood ejected divided by maximally filled left ventricle) show that beetroot juice and potassium nitrate improved exercise capacity presumably via reduced vascular resistance, increased muscle power, and markedly improved vascular compliance (relationship between blood volume of vessel and BP which is generated). Others have shown that synthetic, inorganic sodium nitrite provided by either inhalation or intravenous infusion exerts significant effects on biventricular central filling pressures, cardiac output, and improved exercise capacity. NO-generating nitroglycerin was the first and most frequently used organic nitrate for the clinical treatment of angina pectoris because it causes vasodilatation of the capacitance veins and improves ventricular filling pressure as well as dilates the epicardial coronary arteries; improves coronary blood flow, particularly in ischemic zones; reduces infarct size; and improves clinical outcomes.

Nitrates via synthetic or dietary means, i.e., green leafy vegetables, are pivotal in cardioprotection largely due to NO-induced vasodilation and improved hemodynamics. Cardiac anomalies leading to hypoxia disrupt energetics and mitochondrial function of the heart (Heather et al. 2012). Thus, provision of dietary nitrates may reverse hypoxia-induced effects on respiration, mitochondrial complex I levels, and

activity and oxidative stress that occurs concomitant with hypoxia (Ashmore et al. 2014). Since hypoxia and arginase deficiency (leading to reduced NO) are key features of heart failure, dietary nitrates may confer protection particularly since the non-enzymatic pathway of NO production is preferred at hypoxic sites (Pernow and Jung 2013).

Improved Cognition

Regulation of cerebral blood flow is critical in cognitive function and ischemia and/or energy depletion with NO coupling neural activity to perfusion in the brain (Ogoh 2017). Older adults provided with high-dose or low-dose nitrate displayed significantly increased regional cerebral perfusion in the frontal lobe of the brain involved in executive functioning (working memory, flexible thinking, self-control) (Presley et al. 2011). Moreover, oral nitrate supplementation altered cerebral arterial blood velocity and oxygenation under normoxic conditions but not hypoxic conditions (Fan et al. 2018).

The increased blood flow and regional perfusion in the brain due to beetroot consumption suggests a means of improving mental function and reducing the progression of age-related cognitive decline as well as dementia. Forty healthy adults were randomized to receive placebo or 450 ml beetroot juice (~5.5 mmol nitrate), and after the 90 min consumption period, the bioconversion of nitrate to nitrite was confirmed in plasma, and there is an initial increase in cerebral blood flow. Cognitive performance was also improved revealing that a bolus dose of nitrate could improve cerebral blood flow during task performance and potentially improve cognition. Other studies have demonstrated that dietary nitrate improves oxygenation and cerebral flow during hypoxia (Wightman et al. 2015).

Mitigation of Erectile Dysfunction

Erectile dysfunction is a common, multifactorial disorder associated with aging and is considered a marker for cardiovascular disease suggesting benefit from nitrate-rich food consumption would be logical (Diaconu et al. 2020). NO deficiency is involved in the etiology since it is a pivotal vasodilatory neurotransmitter of penile tissue produced by neural and endothelial cells of the corpora cavernosa with subsequent activation of soluble guanylyl cyclase, which increases cGMP levels releasing calcium from intracellular stores in smooth muscle cells embedded in the vasculature of penile tissue (Diaconu et al. 2020). Recent evidence suggests that neuronal and endothelial NOS (nNOS and eNOS, respectively) exert pivotal roles also via NO bioactivity necessary for erectile function. Moreover, S-nitrosylation/denitrosylation has been shown to regulate eNOS activity via S-nitrosoglutathione reductase contributing directly and indirectly to erectile function/dysfunction (Kavoussi et al. 2019). Circulating plasma nitrate may also serve as a biomarker, in part, for the occurrence or mitigation of ED.

Improvement in Aerobic Exercise Performance

There has been increasing considerable interest in the beneficial effects of dietary nitrate supplementation on athletic performance and exercise in general since dietary nitrates have been described as an ergogenic aid and potential exercise therapeutic (Woessner et al. 2018). Dietary nitrate supplementation may enhance aerobic exercise performance and improve exercise tolerance as reported in numerous studies purportedly via lower oxygen cost during submaximal exercise (Thompson et al. 2014). Other potential mechanisms are greater production of mitochondrial ATP and greater economy in ATP use by skeletal muscles as well as the capacity to significantly enhance vascular function, modulate metabolism, and affect muscle physiology (Ferguson et al. 2013). Acute dietary nitrate supplementation (5 days) can also reduce muscle fatigue primarily caused by reduced contractile capacity (Affourtit et al. 2015).

Dietary nitrates can improve performance during high-intensity exercise. Beetroot juice given as a bolus dose or over several days reduced muscle fatigue and improved exercise performance in intermittent, high-intensity efforts (Domínguez et al. 2018). Nitrate concentration in skeletal muscle is substantially greater than blood concentrations and is subsequently increased by dietary nitrate ingestion suggesting that high-intensity exercise reduces the skeletal nitrate depot following dietary supplementation via the conversion of nitrite to NO (Wylie et al. 2019). The use of nitrate as a biomarker via either its accumulation in plasma or muscle or its depletion via conversion to NO would be useful.

In runners, nitrate-rich whole beetroot consumption acutely improved running performance suggesting optimal function and performance enhancement of track and field athletes. Dietary nitrate supplementation (beetroot concentrate, 140 ml/d; 8 mmol) increased plasma nitrate and reduced VO_2 during submaximal exercise and improved time-trial performance in trained cyclists (Cermak et al. 2012). RSE supplementation (1 g/d; 7 d and 1 h prior) significantly reduced time to completion, increased power and speed, and lowered diastolic BP (Gonzalez et al. 2019). Dietary nitrate supplementation also improved intense intermittent exercise performance (Wylie et al. 2013a, b). Dietary nitrate (beetroot juice, 500 mL/d 4–6 days) acutely reduces BP and the oxygen cost of submaximal exercise for at least 2 weeks in a dose-dependent manner (Lee 2013). Regarding tolerance, beetroot juice amplifies oxygen uptake kinetics and significantly improves exercise tolerance during severe-intensity exercise with elevated metabolic rates (Breese 2013). Nitrates also reduce skeletal muscle metabolism perturbation under conditions of hypoxia. It appears that nitrate supplementation improves exercise tolerance and capacity, which may subsequently improve exercise performance (van de Walle and Vukovich 2018).

Nitrate supplementation improves endurance exercise capacity, but is less effective for time-trial performance (McMahon et al. 2016). Dietary nitrate supplementation can significantly reduce the oxygen cost of exercise and reduce maximal oxygen consumption while maintaining work performance in maximal exercise (Larsen et al. 2007). Dietary nitrate supplementation reduces oxygen cost of low-intensity exercise and enhances tolerance to high-intensity exercise (Bailey et al. 2009). Consumption of nitrate-rich red spinach increases the ventilatory

threshold during graded exercises (Moore et al. 2017). Masschelein et al. show as well that dietary nitrate improves skeletal muscle oxygenation during exercise in hypoxia presumably via NO-induced vasodilation (Masschelein et al. 2012).

Improved Glycemia: Diabetes and Insulin Resistance

There is cumulative evidence that NO is involved in carbohydrate metabolism and lack of NO contributes to the development of type 2 diabetes (Bahadoran et al. 2015). In healthy adults (n = 16) provided with 225 mL beetroot juice, postprandial insulin response was mitigated in the first hour, and the glucose response was reduced in the first half hour (Wootton-Beard et al. 2014). Subjects consuming three beverages including (1) beetroot with lemon; (2) beetroot with glucose, fructose, and sucrose; and (3) beetroot juice with added glucose revealed a positive correlation after beetroot juice plus lemon but not the two other beverages and glycemic response was lower with both the first two glycemic response significantly lower than the last. In a second study, plasma glucose was reduced 35% in subjects consuming the beverage over time and 10% for consumption over a month suggesting that continuous daily consumption is needed (Omar et al. 2016; Olumese and Oboh 2016). Co-ingestion of beetroot juice and glucose resulted in greater increases over 90 min in glucose in obese versus non-obese suggesting obese individuals with an inherently higher risk for developing insulin resistance benefited more than non-obese (Olumese and Oboh 2016). Interestingly, preliminary experimental findings strongly support the hypothesis that nitrate can be considered as a natural anti-obesity agent (Mirmiran 2020).

Directions for Future Research on Nitrates Related to Health

Given the relatively recent appreciation of the capacity of dietary nitrates to generate NO and the clear beneficial effects of NO, there are many areas of research that require attention. First, elucidation of the lowest effective, efficacious dose and the duration needed to significantly lower blood pressure and improve cardioprotection are needed from nutritional intervention studies. Moreover, the demonstration of the potential for these effects in target "at-risk" populations or those with overt chronic disease (hypertensive) is needed. More specific biomarkers with greater sensitivity and validity are needed since plasma NOx are currently measured colorimetrically as nitrite and nitrate, which can be metabolized and/or interconvert. The clear capacity for other dietary components to interact both positively and negatively requires further investigation to elicit dichotomous effects and the microenvironments that may contribute to their action. The ongoing conundrum of whether dietary nitrates cause gastric cancer requires resolution; thus, a reliable risk-benefit analysis would be helpful. Comparison of health or disease status to overall nitrate-rich vegetable, water, and/or dietary supplement intake (amounts and types) and correlation with plasma nitrate and/or nitrite concentrations would be helpful in establishing risk versus benefit. Other recommendations have been made including the development

and/or expansion of dietary databases derived from epidemiological studies to assist with more accurate estimates of dietary consumption patterns and amounts. As such, the cardiovascular benefits may be correlated to intakes. Furthermore, studies of total intake coupled with excretion or at least urinary and plasma concentrations should be incorporated into studies to clarify the disposition of nitrates.

Given the effects of nitrates based largely on the assumption that NO is specifically produced from nitrates and nitrites and that the vasculature is a specific target, then most pathologies and/or conditions that are based in part on vasoconstriction and/or altered hemodynamics should merit additional research. Regarding exercise and sport performance, there is a need for elaborating the impact of dietary nitrates on anaerobic exercise, identifying interactions with other dietary components, and determining efficacious, non-toxic doses and optimal time of supplementation. Collectively, this may help unravel the many noted discrepancies in research results.

Conclusion

Dietary vegetables represent a nitrate-rich source for humans, and consumption can increase plasma levels of nitrate two- to threefold. Both dietary nitrates and nitrites can also be bioactivated endogenously to form NO, as well as other nitrated bioactive molecules, with subsequent reabsorption of 25% back into circulation, which enters the nitrate-nitrite-NO pathway or enterosalivary cycle and is concentrated 10–10,000-fold. The relative critical importance of NO and its vasodilatory function, among many others, have garnered considerable research interest. Research efforts to monitor NO formation and correlate with potential beneficial health outcomes are hindered by the extremely fast half-life of NO. However, the immediate metabolic byproducts of NO formation are nitrate and nitrite. Given the considerable stability of the former, it has been used routinely and effectively in nutritional studies as a surrogate biomarker, although indirect, for NO production. From these studies, dietary nitrates and presumable NO have been correlated with reducing the risk for cerebrovascular incident, myocardial infarction, angina pectoris, hypertension, erectile dysfunction, gastric ulcers, etc. and with improving cardioprotection, nervous system function, and immune function. Given the considerable myriad protective effects, dietary nitrate and nitrite are pivotal in physiological health and homeostasis largely via the formation of NO either enzymatically via NOS or non-enzymatically. Further investigation is needed regarding biological functions, efficacy, dosing, and duration, as well as therapeutic applications of dietary nitrates with a focus on large epidemiological studies.

Applications to Prognosis of Other Diseases or Conditions

NO is a highly reactive, labile, gaseous signaling molecule and free radical produced de novo endogenously and from dietary substrates, viz., nitrates, primarily from leafy green vegetables. It is involved in a variety of physiological and

biochemical processes; thus, its metabolites (nitrate and nitrite) are routinely measured to determine indirectly NO levels and correlated with beneficial health outcomes. NO has a very short half-life of seconds precluding its detection in most nutritional studies, but its immediate metabolites (nitrate and nitrite) have half-lives of hours and days, respectively. Although NO has been associated with virtually every organ system in the body, its most important roles are regulation of blood flow, i.e., hemodynamics, in the vascular endothelium (vascular homeostasis), neurotransmission, and host defense mechanisms. These three systems affect most organ and tissue environments in the body; thus, detection of nitrates is applicable to most if not all disease conditions in some aspect. In particular, the capacity of NO as a potent vasodilator and the omnipotence and importance of vasculature for nutrition, waste removal, and oxygen delivery in all respiring tissues support the notion of its importance in health and disease depending on concentration. Nitrates and nitrites presumably via NO production affect myriad biochemical processes in cardiovascular health, nervous system function, pulmonary function, inflammatory processes, immune system robustness, and genitourinary physiology. As such, plasma or tissue nitrate concentrations may be used as a biomarker of diagnosis, prognosis, therapeutic efficacy, and effectiveness of dietary or nutritional intervention.

Mini-Dictionary of Terms

Griess Method: The Griess method is a colorimetric analytical test for the presence of aqueous nitrite ion in biological fluids used routinely in nutritional studies. Nitrite is detected and analyzed by the formation of a red color upon treatment of a nitrite-containing sample with the Griess reagent, which consists of 2% sulfanilamide in 5% phosphoric acid and 0.2% N-(1-naphthyl)ethylenediamine.

Nitrate: A polyatomic ion (NO_3) in the diet containing nitrogen and oxygen often associated with cured meats, water, and green, leafy vegetables. Nitrates can lead to the production of bioactive NO in the body.

Nitric Oxide (NO): A polyatomic gaseous free radical produced by most every type of cell in the human body. NO is crucial for blood vessel health and as a potent vasodilator causing the vasculature to widen and increase blood flow and lower blood pressure.

NOx: The generic term for a group of highly reactive gases, all of which contain nitrogen and oxygen in varying amounts [such as **nitric oxide** (**NO**) and **nitrogen dioxide** (**NO_2**)] and more frequently in nutritional studies as nitrite and nitrate, the immediate metabolites of nitric oxide.

Nitrite: Nitrite is a polyatomic compound with the formula NO_2 that occurs naturally in the human body and in some foods as they are added to processed foods to extend shelf life. Nitrite can interconvert to nitric oxide, dilate your blood vessels, and lower blood pressure conferring myriad health benefits and enhancing physical performance.

Key Facts of Nitrates

Nitrates are prevalent in the human diet due to agricultural and agronomic practices.

Nitrates are particularly prevalent in leafy green vegetables and other plants such as beetroot and red spinach and can increase plasma levels by two- to threefold.

Nitrates enter peripheral circulation where interconversion to nitrite and nitric oxide, a potent vasodilator, can occur.

NO as a molecule is extremely labile with a very short half-life, i.e., seconds.

Nitrate and nitrite are the two most prevalent immediate metabolites of NO with the former being stable in plasma for days and the latter hours.

Nitrates in biological fluids and compartments can be used as a surrogate biomarker for NO production and correlated with potential beneficial effects of nitrate consumption.

Summary Points

Dietary nitrates and nitrites are distributed in the food supply in substantial amounts and particularly in leafy green vegetables.

Upon consumption, nitrates are readily absorbed, and levels increase in the bloodstream entering the enterosalivary recirculation pathway.

Nitrates and nitrites can form nitric oxide, a potent vasodilatory molecule produced normally endogenously.

Plasma levels of nitrates can be detected and correlated to the reduction of risk factors for disease and the improvement of exercise and athletic performance.

Nitrate, the most stable immediate metabolite of NO production and metabolism, can readily be measured in plasma and other biological compartments as a surrogate biomarker for NO production.

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Blood and Urinary Flavonoids

6

Methods of Analysis and Applications

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Abstract

Flavonoids are a large and complex group of phenolic compounds present in plant origin foods that may promote health and prevent diseases in humans. Traditional methods to estimate flavonoids intake are based on self-reported questionnaires and food composition databases. These instruments not only are fast, cheap, and practical, but also have certain limitations when estimating the real dietary exposure to flavonoids. However, measurement of flavonoids and their metabolites in biological fluids such as blood and urine may overcome these limitations. Although there are different analytical methods and instruments that can identify and quantify the different flavonoid subclasses, both liquid and gas chromatography coupled or not to mass spectrometry are, currently, the most commonly used methodologies. This chapter summarizes the current literature on these and other innovative technologies for the analysis of flavonoids in human biospecimens, as well as their application in nutritional studies evaluating the exposure to these compounds and their relationships with diseases.

Keywords

Flavonoids · Biomarkers · Methods · Analysis · Urine · Plasma · Serum · Sample preparation · Chromatography · Mass spectrometry

Abbreviations

APCI	Atmospheric Pressure Chemical Ionization Source
APPI	Atmospheric Pressure Photoionization
BHT	Butylated hydroxytoluene
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CVD	Cardiovascular disease
DBS	Dried blood spot cards
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
EKC	Electrokinetic chromatography
ESI	Electrospray Ionization Source
FFQ	Food frequency questionnaire
GC	Gas Chromatography
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
QDF	Quantitative dietary fingerprinting
SPE	Solid-phase extraction

T2D	Type 2 diabetes
TR-FIA	Time-resolved fluorescence immunoassay
UHPLC	Ultra-high-performance liquid chromatography
USDA	United States Department of Agriculture

Introduction

Chemical Composition and Bioactivity

Flavonoids are a class of phenolic compounds widely present in the plant kingdom. These compounds, which are derived from secondary metabolism, are widely distributed in different tissues and organs of plants, acting as regulators of plant development, pigments, and defense against ultraviolet radiation and pathogen infection, signaling mediators between plants and microorganisms, nodulation, and pollen fertility (Falcone Ferreyra et al. 2012). Currently, over 6000 naturally occurring flavonoids have been characterized from various plants, and this number continues to grow (Panche et al. 2016). Chemically, flavonoids consist of 15 carbon atoms arranged in the form C6-C3-C6, consisting of two benzene rings (A- and B-rings) linked by a heterocyclic 3-carbon unit (C-ring) (Fig. 1). Flavonoids are usually subdivided into different subclasses according to their chemical structure, and the degree of unsaturation and oxidation of the C-ring. The main flavonoid subgroups are anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols, and isoflavones (Fig. 1).

Numerous experimental studies have shown that flavonoids have many therapeutic properties, including antioxidant, anti-inflammatory, antimicrobial, and immunemodulatory (Zamora-Ros et al. 2014). In addition, recent meta-analyses suggest that a high dietary intake of total flavonoids and flavonoid subclasses may protect against the risk of cardiovascular disease (CVD), some cancers, and type 2 diabetes (T2D) (Del Bo' et al. 2019). Moreover, it was recently suggested that flavonoids may be used for the treatment of some neurodegenerative disorders, especially for Alzheimer's disease and dementia (Kaur et al. 2022).

Food Sources, Intake, and Metabolism

Flavonoids are widely distributed in plant origin foods of the human diet. Some of the main dietary sources of flavonoids include fruits, vegetables, nuts, seeds, chocolate, tea, and red wine (Table 1) (Zamora-Ros et al. 2016). In fact, flavonoid composition largely varies in type and concentration between similar food sources, where they sometimes play an important role in food-sensorial aspects related to aroma, taste, and color (Lesschaeve and Noble 2005).

The intake of total flavonoids and flavonoid subclasses greatly differs between individuals and populations due to the consumption of different dietary patterns and foods. The mean intake of total flavonoids worldwide, without considering thearubigins, was estimated around 400 mg/day, ranging from 150 mg/day in Latin-

Fig. 1 General chemical structures of flavonoid subclasses

Table 1 Examples of common flavonoid compounds and their main food sources

Flavonoid subgroup	Representative compounds	Main dietary sources
Anthocyanidins	Cyanidin Delphinidin Malvidin Peonidin Petunidin	Bilberries, blueberries, blackberries, black currants, chokeberry, cranberries, pomegranate juice, red grapes, and strawberries
Flavan-3-ols	Monomers: (+)-catechin (−)-epicatechin Oligomers and polymers: Proanthocyanidins	Apples, bananas, blueberries, dark chocolate, pears, red wine, and tea
Flavanones	Hesperetin Eriodictyol Naringenin	Citrus fruits and citrus-based juices, such as orange, grape, lemon, lime, and tangelo
Flavones	Luteolin Apigenin	Celery, chamomile, *Ginkgo biloba*, mint, parsley, red peppers, and thyme
Flavonols	Kaempferol Myricetin Quercetin	Apples, berries, grapes, onions, lettuce, red wine, tea, and tomatoes
Isoflavones	Daidzein Genistein Glycitein Biochanin A	Legumes, mainly soybeans and soy-derived foods

American countries to 600 mg/day in Australia and the UK (Escobar-Cévoli et al. 2017). Flavan-3-ols are, by far, the most consumed flavonoid subclass (70–75%). In general, women and older people with healthy lifestyles and dietary habits, and a higher educational level and socioeconomic status, tend to have a higher total flavonoid intake (Escobar-Cévoli et al. 2017).

Once ingested, flavonoid absorption is largely variable depending on their chemical structure. It has been estimated that absorption is high for isoflavones and flavonols, and low for catechins, flavanones, and anthocyanins (Hollman 2004). Flavonoids, except catechins, are mostly present in foods as glycoside forms, this is conjugated to a sugar. Glycosides are mainly absorbed after enzymatic hydrolysis from sugar moieties by hydrolases at the intestinal brush border. Unabsorbed flavonoids reach the colon and are hydrolyzed by the microbiota to low-molecular weight metabolites called phenolic acids and lactones, some of which may be absorbed, and others eliminated in the feces. They can also be transported into enterocytes, where the sugar moieties are removed by β-glucosidases. This is the first and determinant step in the absorption of flavonoids (Murota et al. 2018; Pei et al. 2020). Once flavonoid aglycones enter the intestinal epithelial cells, phase II enzymes produce the corresponding conjugated metabolites (e.g., glucuronide, sulfate, and/or methylate derivatives). After intestinal conjugation, flavonoid metabolites are transported to the portal vein. Once in the liver, they can undergo a new

conjugation via phase II enzymes. Elimination in the bile is, quantitatively, the most important route of flavonoid elimination, especially as conjugated metabolites. However, the glucuronide and sulfate conjugates of the methyl esters are also excreted in urine (Wen et al. 2017).

Measurement of Exposure

Traditionally, the intake of flavonoids and other phenolic compounds in epidemiological studies is measured by combining the use of dietary self-report questionnaires and food composition tables or databases. The most common dietary questionnaires used in prospective cohort studies include food frequency questionnaires (FFQ), 24-h dietary recalls, and food diaries, while the most complete and commonly used flavonoid databases to date are Phenol-Explorer (Neveu et al. 2010) and the USDA Database for the Flavonoid Content of Selected Foods (Bhagwat and Haytowitz 2016). Strengths and limitations of these instruments have been discussed elsewhere (Xu et al. 2021). In general, self-report questionnaires not only offer a practical and affordable way to record a person's habitual diet, but may also introduce bias in the flavonoid intake assessment due to imprecise reporting of the real amount consumed; moreover, databases do not consider the variability of food composition and flavonoid bioavailability. To overcome such limitations, currently, there is the possibility of using objective nutritional biomarkers, defined as small molecules present in biological fluids reflecting exposure to specific foods or food components. Nutritional biomarkers of flavonoids are essential to accurately estimate their intake and properly investigate their potential beneficial health effects (Zamora-Ros et al. 2012).

Studies focused on searching for flavonoid nutritional biomarkers; their bioavailability and pharmacokinetics are mainly carried out in whole blood, plasma, serum, and urine. Urine is extensively used because it is a noninvasive biological fluid in which it is easy to monitor flavonoid concentrations (Delanghe and Speeckaert 2014); however, plasma and serum are the biological matrices most commonly used in epidemiological studies. These are preferably used over the whole blood because they are more stable, easy to process and analyze (Zamora-Ros et al. 2012). In the near future, dried blood spot (DBS) cards could continue to be increasingly used as an alternative for monitoring flavonoid metabolites in blood samples obtained by a finger-prick lancet blotted onto filter paper, and, therefore, avoiding the use of needles in a blood draw (Yuste et al. 2018).

Regardless of the type of biospecimen and its handling, the inter- and intravariability in flavonoid concentrations need to be considered as a potential limitation of their use as biomarkers. Currently, the main analytical techniques used for the analysis of flavonoids in human urine and blood are based on both liquid and gas chromatography (LC and GC, respectively) either coupled or not to mass-spectrometry (MS). Despite the use of other techniques (such as those described later in this chapter), LC continues to be used today as the gold standard technique due to its high capacity to determine dozens of small molecules in humans coming from endogenous metabolism and exogenous sources.

Sample Preparation

Prior to their analysis, biological samples often undergo a series of pretreatment strategies in order to purify and concentrate the compounds of interest by removing interfering biological matrix components, such as salt content in urine, or proteins in plasma and serum. Figure 2 summarizes some of the more common sample pretreatment steps commented in more detail below.

An *adjustment of pH* is fundamental, especially before enzymatic hydrolysis in order to ensure the enzyme's optimum pH. To achieve the target pH, a buffer

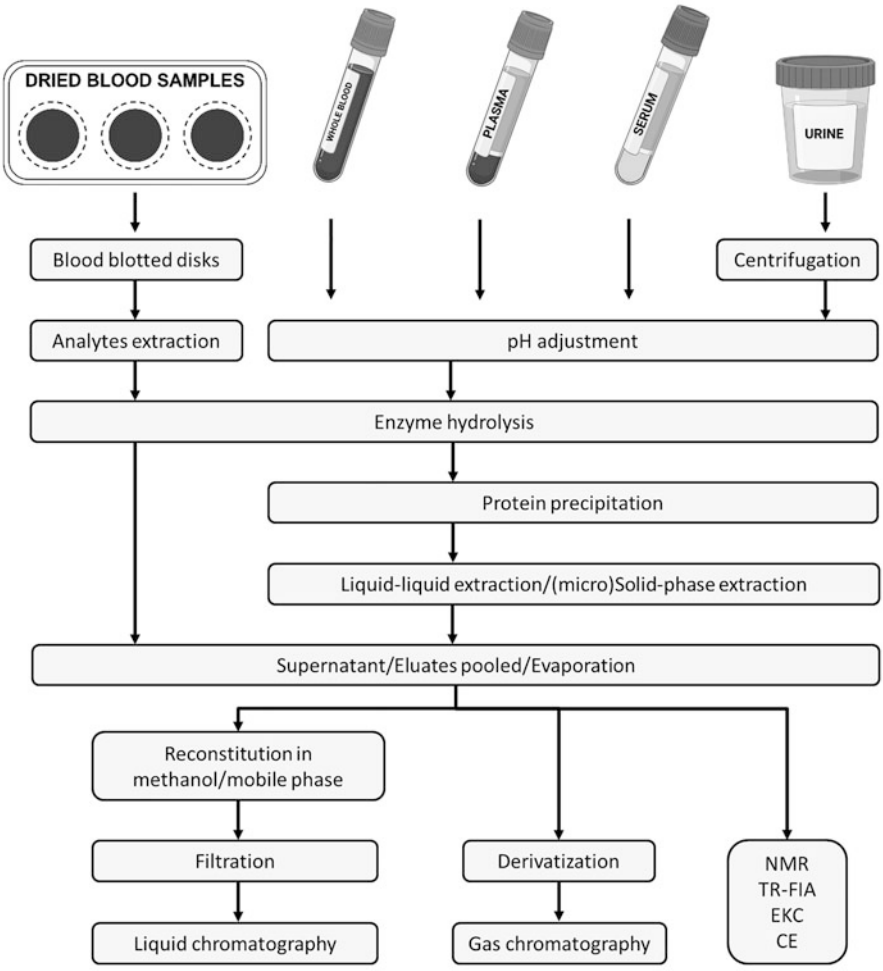

Fig. 2 Pretreatment workflow for flavonoid analysis in blood, plasma, serum, urine samples, and blood spot cards. Abbreviations: CE, capillary electrophoresis; EKC, electrokinetic chromatography; NMR, nuclear magnetic resonance; TR-FIA, time-resolved fluorescence immunoassay

commonly composed of sulfate, sodium, and phosphate salts is used (Quifer-Rada et al. 2017). Additionally, for plasma and serum samples, an antioxidant is usually added (e.g., ascorbic acid, butylated hydroxytoluene (BHT), or ethylenediaminetetraacetic acid (EDTA)) (Quifer-Rada et al. 2017). For urine samples, before pH adjustment, an initial centrifugation is usually performed to remove particulate material, which is crucial to obtain optimal analytical results (Stevens et al. 2019).

Enzymatic and chemical hydrolysis pursues the separation or deconjugation of sulfate and glucuronide moieties from the flavonoid aglycone. The most common enzymes used are β-D-glucuronidase and sulfatase, both used under a broad range of temperatures and time conditions, ranging from 37 °C to 55 °C, and from 30 min to 24 h, respectively. Both enzymes can be used individually or in combination.

The *protein precipitation* step is usually performed afterward the hydrolysis. In some cases, protein precipitation is carried out before enzyme hydrolysis, but then a pH adjustment after protein precipitation will be needed. Polar solvents (e.g., acetonitrile, methanol, aqueous trichloroacetic acid, acetic acid, and acetone) or organic acids (e.g., phosphoric acid, perchloric acid, aqueous formic acid, hydrochloric acid, trifluoroacetic acid, dimethylformamide, and dimethyl sulfoxide) are commonly used for protein precipitation (López-Yerena et al. 2021). The *sample acidification*, due to the reagents indicated above, decreases the protein-phenol bond, resulting in a better overall extraction recovery. Protein precipitation is aided and enhanced via the use of vortex or ultrasonication (less common) and then centrifugation (Pereira-Caro et al. 2016). Once the protein has been precipitated, the supernatant is collected, concentrated, and resuspended in a new solvent, although sometimes it is used directly in the next step.

The *cleanup* step involves concentration and purification procedures, which are usually performed via techniques based on liquid-liquid extraction (LLE) or solid-phase extraction (SPE). LLE involves the adsorption of the compounds present in the sample into an organic solvent, mainly: ethyl acetate, diethyl ether, and methanol (López-Yerena et al. 2021). Although LLE has reduced the volume of solvent used across time, currently, it is not commonly used. Analogous to protein precipitation, reagents used for LLE are mixed with the biological fluid, then the sample is vortexed, centrifuged, and, in some cases, ultrasonicated (Breiter et al. 2011). The resultant supernatant is usually concentrated to dryness and resuspended in another solvent ready to inject (usually the mobile phase, and, if not, methanol or an aqueous methanol solution are commonly used) or submitted to the next step in the pretreatment protocol. SPE also involves the adsorption of the analytes from the biological matrix onto a solid sorbent, and subsequent elution of the analytes from the sorbent into an organic solvent that may be injected into the LC system for analysis or used in a subsequent step. Commercial SPE products are available such as cartridges, tubes, and 96-well plates (μ-SPE). Advantages of SPE over LLE include low solvent consumption, ease of use, efficient analyte extraction, and automation capability (López-Yerena et al. 2021).

In the particular case of DBS cards, once a finger-prick lancet is blotted onto filter paper and dried, the filter paper is punched out around the blood circle (disks) and eluted with solvent/buffer followed by enzymatic hydrolysis and the LLE step, as described above (Melby et al. 2005), or could also undergo no further pretreatment before injection (Yuste et al. 2018).

Separation and Detection Techniques

Liquid Chromatography and Mass Spectrometry

Analytical techniques based on LC, such as the high- and ultra-high-performance liquid chromatography (HPLC and UHPLC, respectively) are, by far, the techniques most used to separate, identify, and quantify dietary flavonoids from human biofluids. The separation occurs based on the interaction of the compounds present in the sample with a mobile and a stationary phase. Once separated, the compounds enter a detector, a device that provides an electronic signal proportional to the concentration of the eluting compounds. Although there are many different detectors, the most commonly used in LC systems include ultraviolet (UV), fluorescence, and electrochemical detectors. Overall, HPLC and UHPLC are similar in accuracy and precision, but UHPLC offers higher resolution, greater sensitivity, and faster analysis time. Due to these advantages, UHPLC is becoming more and more popular in laboratories, especially for complex sample analysis.

Mass spectrometry (MS) is a technique based on the conversion of the analyte molecules into a charged (ionized) state, with the subsequent analysis of ions and ion fragments produced during the ionization process, based on their mass-to-charge ratio (m/z). Although, there are several types of ionization, the most commonly used for flavonoid analysis is still the electrospray ionization (ESI), followed by atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). Nowadays, the coupling of LC with MS (LC-MS) has the potential to simultaneously analyze dozens to hundreds of chemical species.

Isoflavones. Isoflavones are probably the most frequently analyzed flavonoid subclass, including daidzein, genistein, glycitein, and their microbial metabolites equol and *O*-desmethylangolensin, which can be individually or simultaneously measured in urine and plasma samples via HPLC and UHPLC systems (van der Velpen et al. 2014; Ahn-Jarvis et al. 2015; Redruello et al. 2015; Ideno et al. 2018). Sixteen isoflavones, including glucuronide and sulfate-conjugated metabolites of daidzein and genistein, were simultaneously determined in human urine and plasma samples using an HPLC system coupled to a UV detector (Hosoda et al. 2011). Daidzein, genistein, and equol have also been detected and measured in DBS samples stored at 4 °C and 25 °C for 8 weeks using HPLC with an electrochemical detector (Melby et al. 2005). Currently, the use of HPLC-MS or UHPLC-MS allows the determination of free and conjugated forms (i.e., sulfates, glucuronides, and sulfoglucuronides) of genistein, dihydrogenistein, glycitein, daidzein, dihydrodaidzein, and the microbial metabolites *O*-desmethylangolensin and equol in urine (Soukup et al. 2014; Saha and Kroon 2020), plasma (Soukup et al. 2016; Obara et al. 2019; Kim et al. 2020), serum (Chan et al. 2006; Kano et al. 2006), and DBS (Cao et al. 2009). Biochanin A and biochanin B, two bioactive isoflavone derivatives isolated from red clover, were recently identified and quantified in urine, plasma, and serum samples by HPLC-MS and UHPLC-MS (Zhou et al. 2020; Ávila-Gálvez et al. 2021).

Flavan-3-ols. Following isoflavones, flavan-3-ols are the most widely studied flavonoid subclass in human urine and blood samples via LC and LC-MS

techniques. The monomers (+)-catechin and (−)-epicatechin, and their corresponding galloyl and gallated derivatives, are usually separated by HPLC in urine, plasma, and serum samples, and identified by a wide variety of detectors. For example, a very sensitive method based on capillary LC with electrochemical detection was able to quantify attomole quantities (1×10^{-18}) of (−)-gallocatechin gallate, (−)-epicatechin, (−)-epigallocatechin 3-gallate, (−)-epicatechin 3-gallate, and (−)-catechin gallate in human plasma (Kotani et al. 2007). Native and conjugated forms of (+)-catechin and (−)-epicatechin, including glucuronides, sulfates, and methyls, are usually identified in urine and plasma samples by using HPLC-MS and UHPLC-MS methodologies commonly with an ESI interface operating in negative ionization mode (Urpi-Sarda et al. 2009b; Del Rio et al. 2010; Saha et al. 2012; Actis-Goretta et al. 2012; Park et al. 2018; Hakeem Said et al. 2020). Procyanidins are homo-oligomeric forms of (+)-catechin and (−)-epicatechin and due to their variable degree of polymerization (i.e., 2–10 units), procyanidins are poorly absorbed in humans and thus rarely found in their native form in blood or urine samples. Although their separation can be easily achieved by HPLC and UHPLC, the detectors commonly coupled to these systems are, in general, incapable of distinguishing between the different procyanidin types. The coupling of MS to LC has improved the elucidation of their structure; however, the identification of procyanidins in human urine, plasma, and serum is still limited (Sano et al. 2003; Urpi-Sarda et al. 2009a). Over the last decade, an increasing number of nutrition studies have described the use of HPLC-MS and UHPLC-MS for the analysis of compounds derived from the microbial metabolism of monomeric and polymeric forms of flavan-3-ols. These compounds, namely phenyl-γ-valerolactones and phenylvaleric acids, are more frequently detected in urine than in plasma (Boto-Ordóñez et al. 2013; Wiese et al. 2015; Brindani et al. 2017; Anesi et al. 2019; Hollands et al. 2020; Hakeem Said et al. 2020).

Flavonols. Detection of quercetin, kaempferol, and isorhamnetin can be achieved by using an HPLC with a UV detector (Sadeghi et al. 2021). Furthermore, they can be detected via ESI+ mode in urine and plasma samples via HPLC and UHPLC coupled to MS (Mullen et al. 2004; Ding et al. 2006; Rodriguez-Mateos et al. 2016).

Flavanones. Concentrations of hesperetin and naringenin can be measured in urine and plasma via HPLC (Barfi et al. 2013). Similarly, glucuronide, sulfate, and *O*-glucuronyl-sulfate derivatives of hesperetin, naringenin, eriodyctiol, homoeriodictyol, and narirutin can be determined, in clinical trials, in human urine (Aschoff et al. 2016; Zeng et al. 2017; Pereira-Caro et al. 2017; Agulló et al. 2021a, b), and in plasma (Mullen et al. 2008; Lévèques et al. 2012; Pereira-Caro et al. 2016) using HPLC-MS and UHPLC-MS with an ESI interface operating in negative-ionization mode.

Anthocyanidins. Advances in LC-MS techniques have allowed the identification and quantification of a large number of anthocyanidins in human biofluids. The compounds forming this subclass (e.g., cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) are commonly separated by HPLC and UHPLC and identified by MS with ESI in a positive ionization mode. By using these techniques, free (anthocyanidins) and glycosidic forms (anthocyanins) (i.e., galactoside, glucoside, and arabinoside), as well some phase II conjugates

(i.e., glucuronide, sulfate, and methyl) of anthocyanidins, have been determined from human urine (Cooke et al. 2006; Kalt et al. 2014, 2017; Kasote et al. 2019; Agulló et al. 2020; Kaiser et al. 2020) and plasma (Cooke et al. 2006; Kasote et al. 2019; Kaiser et al. 2020) after the acute consumption of test meals.

Flavones. To date, there are a few different LC-MS methodologies for the specific analysis of flavones in human blood and urine. Following the intake of different doses of pure baicalein, baicalein and baicalin were detected in human urine (Li et al. 2014; Pang et al. 2016b) and plasma (Li et al. 2014; Pang et al. 2016a, b) by using HPLC-MS with positive ESI. In addition to baicalein, five metabolites, including baicalein 6,7-di-*O*-glucuronide, baicalein 7-*O*-glucuronide, 6-methoxybaicalein 7-*O*-glucuronide, baicalein 6-*O*-glucuronide, and 7-methoxybaicalein 6-*O*-glucuronide, were also detected in plasma via HPLC-MS-ESI (Guo et al. 2011). The urinary excretion of 31 different metabolites of hypolaetin, methylhypolaetin, isoscutellarein, methylisoscutellarein, and apigenin was quantified using an HPLC-MS-ESI method validated for this purpose (Petreska Stanoeva and Stefova 2013).

The simultaneous determination of different flavonoid subclasses can also be achieved via LC-based methods. For example, representative compounds belonging to isoflavones (daidzein and glycitein), flavan-3-ols ((+)-catechin and (−)-epicatechin), and flavonols (rutin and quercitrin) can be separated from urine samples by UHPLC and detected by UV detectors (Baranowska and Magiera 2011). Nevertheless, an increasing number of studies report the use of LC-MS methodologies for the simultaneous determination of compounds belonging to two or more flavonoid subclasses in human urine and blood. Serum concentrations of genistein, daidzein, glycitein, equol, biochanin A, biochanin B, quercetin, kaempferol, luteolin, and naringenin were analyzed from serum samples via HPLC-MS-ESI methodology (Palma-Duran et al. 2015). Two highly sensitive methods based on differential isotope labeling with ^{13}C- and ^{12}C-dansyl chloride were developed and validated by Achaintre et al. (2016, 2018) for the analysis of 38 structurally diverse polyphenols in urine and plasma, respectively. These methods, which used a UHPLC-ESI-MS/MS platform, enabled the identification and quantification of 13 flavonoids, including three flavonols (kaempferol, quercetin, and isorhamnetin), one flavone (apigenin), two flavanones (naringenin and hesperetin), three isoflavones (daidzein, genistein, and equol), one dihydrochalcone (phloretin), and three flavan-3-ols ((+)-catechin, (−)-epicatechin, and (+)-gallocatechin) (Achaintre et al. 2016, 2018) in both urine and plasma samples. Recently, González-Domínguez et al. (2020) developed and validated a UHPLC-ESI-MS/MS method for the simultaneous identification and quantification of about 350 food-derived metabolites in human urine. This "quantitative dietary fingerprinting" (QDF) included a large number of flavonoids, including free and phase II-conjugated forms of catechins ((+)-catechin and (−)-epicatechin), flavanones (naringenin and hesperetin), isoflavones (daidzein, genistein, biochanin A, and biochanin B), flavones (apigenin and luteolin), flavonols (quercetin, isorhamnetin, and kaempferol), chalcones (phloretin), anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin), and some direct microbe-derived flavonoid metabolites (e.g., phenyl-γ-valerolactones, phenylvaleric acids, and equol) (González-Domínguez et al. 2020).

Gas Chromatography and Mass Spectrometry

Gas chromatography (GC) is a technique capable of separating highly complex mixtures based primarily upon differences in boiling point/vapor pressure and polarity (Stauffer et al. 2008). Coupled to mass spectrometry (MS) and performing a derivatization process, GC has allowed the study of six flavonoid subclasses: isoflavones, flavonols, flavones, flavanones, flavan-3-ols, and dihydrochalcones. Daidzein and its microbial metabolites equol and *O*-desmethylangolensin, genistein, and glycitein are, by far, the isoflavones most commonly analyzed by GC-MS in urine (Xu et al. 2000; Grace et al. 2003; Moors et al. 2007), plasma (Howes et al. 2002), and serum (Pumford et al. 2002). Along with these compounds, novel isoflavone derivatives including tetrahydrodaidzein, 6′-hydroxy-*O*-dimethylangolensin, 2-dihydro-*O*-dimethylangolensin, dihydrodaidzein, and cis/transisomers of tetrahydrodaidzein were characterized and identified by GC-MS (Joannou et al. 1995). Using GC-MS, four novel isoflavone metabolites (i.e., 3′,4′,7-trihydroxyisoflavone, 4′,6,7-trihydroxyisoflavone, 4′,7,8-trihydroxyisoflavone, and 3′,4′,5,7-tetrahydroxyisoflavone) were identified in urine collected after soy supplementation (Joannou et al. 1995). Compared with isoflavones, flavan-3-ols are less frequently analyzed in blood and urine by GC-MS. Nowadays, in fact, most GC-MS methods are capable of analyzing more than one flavonoid subclass (Soleas et al. 2001; Kahle et al. 2011; Wang et al. 2012). Advances in metabolomics based on GC-MS have additionally allowed the urinary and plasma detection of microbial metabolites derived from the intake of (−)-epicatechin and procyanidin B1, including hydroxyphenyl-valerolactone and hydroxyphenyl-valeric acid compounds (Wiese et al. 2015). Among flavonols, only quercetin and kaempferol have been detected in plasma, serum, and urine samples via GC-MS techniques (Watson and Oliveira 1999; Soleas et al. 2001; Goldberg et al. 2003; Kahle et al. 2011). Minor flavonoid compounds, such as flavones and flavanones, have also been detected in human blood and urine by GC-MS. A specific GC-MS methodology was developed to detect and quantify icaritin and desmethylicaritin, two bioactive prenylflavones found in traditional Chinese medicinal herbs (Shen et al. 2007). Diosmetin and hesperetin, both aglycones of the flavone glycoside diosmin and the flavanone glycoside hesperidin, were simultaneously determined by a GC-MS method in human plasma (Pereira-Caro et al. 2014).

Capillary Electrophoresis

Capillary electrophoresis is a fast and simple analytical technique to separate ions based on their electrophoretic mobility. Chiral capillary electrophoresis was successfully applied for the detection of (+)-catechin and (-)-epicatechin in human urine and plasma (el-Hady and el-Maali 2008; el-Hady et al. 2008). A less common analytical methodology was used for isoflavones in human serum (Starkey et al. 2002), consisting of capillary electrochromatography (CEC) and photodiode array detector. Researchers separated daidzin, glycitin, genistin, daidzein, glycitein, genistein, acetyl daidzin, acetyl genistin, and malonyl genistin, through a highly efficient solute

separation, with sensitivity at low levels and rapid analysis (10–15 min runs). Serum levels of isoflavone were also measured in volunteers who were supplemented for 4 days with soy products. The amounts of genistein and daidzein increased over the supplementation time, demonstrating the effectiveness of CEC methods for clinical studies (Starkey et al. 2002).

Electrokinetic Chromatography

Electrokinetic chromatography (EKC) is a mode of capillary electrophoresis that permits the separation of electrically neutral molecules (Starkey et al. 2002). Micellar electrokinetic chromatography (MEKC) has become the most popular technique among various EKC modes for the analysis of pharmaceuticals (Starkey et al. 2002). A sweeping-MEKC by long alkyl chain-ionic liquid was applied for the determination of the following tea catechins in human plasma: gallocatechin, (+)-catechin, (-)-epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, and epigallocatechin (el-Hady and Albishri 2014).

Nuclear Magnetic Resonance

An SPE-preparative LC-MS-NMR workflow was successfully implemented for preparative isolation of conjugated valerolactone metabolites of catechin-based polyphenols from the urine of black tea consumers (el-Hady and Albishri 2014). This technique was able to detect and quantify the amounts of five different conjugated dihydroxyphenyl-γ-valerolactones, including glucuronides and sulfates, in urine samples.

Time-Resolved Fluorescence Immunoassay

Immunoassay offers potential advantages compared with other methods, such as the use of a small sample volume, direct sample testing, faster, simplicity, and low cost (Talbot et al. 2007). Time-resolved fluorescence immunoassay (TR-FIA), which applies dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) enhancement solution to develop the fluorescence, has allowed the assessment of isoflavones in both plasma and urine samples (Uehar et al. 2000; Wang et al. 2000; Brouwers et al. 2003; Talbot et al. 2007). This technique presented high sensitivity with a detection limit of 1.8 pg/20 mL for plasma daidzein; 3.1 pg/20 mL for plasma genistein (Wang et al. 2000); and 3.9 nmol/L for urinary daidzein, 88.8 nmol/L for urinary genistein, and 2.2 nmol/L for urinary equol (Talbot et al. 2007). The specificity of TR-FIA is considered excellent because the method yields results similar to the GC-MS method (Uehar et al. 2000; Wang et al. 2000; Brouwers et al. 2003). Thus, TR-FIA has been used in an intervention study to measure isoflavone compliance (Talbot et al. 2007).

Applications of Flavonoid Biomarkers

Assessment of the Effect on Disease Risk

Epidemiological evidence has suggested several potential beneficial relationships between dietary flavonoids intake and the risk of obesity, diabetes, CVD, certain types of cancer, and neurodegenerative diseases (Del Bo' et al. 2019). Furthermore, experimental in vitro and in vivo research has shown beneficial effects against intestinal inflammation (Vezza et al. 2016) and viral infections (Zakaryan et al. 2017). Most studies focused on flavonoid-disease associations used dietary questionnaires to estimate flavonoid intake; however, there is increasing number of studies using blood and urinary levels of flavonoids as an indicator of their exposure. For example, higher urinary excretion of total isoflavones and daidzein were related to an increase in CVD mortality (Reger et al. 2016). Conversely, an intervention with a 25 g/day intake of fermented soy powder reduced total and low-density lipoprotein (LDL) cholesterol by approximately 7% in individuals with high CVD risk (Jung et al. 2021). In the same study, higher concentrations of daidzein, genistein, and glycitein were detected in the group of participants receiving fermented soy, compared to their control counterparts. Systolic and diastolic blood pressure were inversely associated with urine flavan-3-ols concentrations in general, based on the 5–3′,4′-dihydroxyphenyl-γ-valerolactone metabolite, and one specific for (−)-epicatechin intake, based on structurally related (−)-epicatechin metabolites (SREM) (Ottaviani et al. 2020). Moreover, these biomarkers of flavan-3-ols demonstrated an improvement in the blood lipid profile, but no association with CVD incidence or mortality was observed (Ottaviani et al. 2020). In an intervention study in male participants, plasma quercetin-3-*O*-ß-D-glucuronide from cranberry juice was positively correlated with an improvement in both flow-mediated dilation and vascular function (Rodriguez-Mateos et al. 2016). Plasma concentrations of total flavanones after the acute intake of orange juice were not significantly associated with cardiovascular risk biomarkers (i.e., blood pressure, endothelial function, central arterial stiffness, cardiac autonomic function, platelet activation, and oxidase-dependent elimination of endothelial nitric oxide gene expression) in men with moderate risk of CVD (Schär et al. 2015). Furthermore, a Spanish study reported lower levels of oxidized-LDL after an intervention with cocoa supplementation within a hypocaloric diet (Ibero-Baraibar et al. 2014), while the urinary flavonoid metabolites 2,5,7,3′,4′-pentahydroxyflavanone-5-*O*-glucoside and 7,4′-dimethoxy-6-C-methyl-flavanone were also more excreted in the cocoa-supplemented group (Ibero-Baraibar et al. 2016). Urinary levels of kaempferol and daidzein, but not quercetin, isorhamnetin, tamarixetin, genistein, and apigenin, were inversely associated with acute coronary syndrome risk (Bredsdorff et al. 2013). Few studies have assessed the role of blood and urinary isoflavones in T2D risk. Evidence from case-control studies showed a negative inverse association between concentrations of daidzein and genistein and T2D (Ko et al. 2014; Ding et al. 2015). However, these results

were not supported by a later cohort study (Dong et al. 2020). The anticarcinogenic effects of flavonoids are partially explained by their antioxidant activity, their ability to inhibit cell proliferation, and angiogenesis (Nijveldt et al. 2001). Moreover, due to their structural similarity to 17-β-estradiol, isoflavones are also called phytoestrogens. Isoflavones may bind to both estrogen receptors (ERα and ERβ), showing a higher affinity for Erβ, which participate in proliferation inhibition and apoptosis stimulation (Ziaei and Halaby 2017). Most flavonoid biomarker-based research has focused on isoflavones and their possible protection against sex hormone–related cancers. Such evidence, including 22 epidemiologic studies, has been previously summarized, and the authors concluded that there was no association of blood isoflavone concentrations with prostate cancer, but there were protective effects against breast cancer in Asian populations (Zamora-Ros et al. 2014). Other flavonoids, such as isorhamnetin, kaempferol, flavanone, naringenin, and quercetin, have shown beneficial effects against breast cancer development (Feng et al. 2021; Sadeghi et al. 2021). No associations have been found for urinary genistein and luteolin with colorectal cancer mortality, recurrence, and disease-free survival (Jiang et al. 2019), urinary isoflavone and precancerous intestinal lesions (Polimeno et al. 2020), and primary liver cancer risk (Michikawa et al. 2015), as well as between blood polyphenols, including flavonoids, and colon cancer risk (Murphy et al. 2018) and differentiated thyroid cancer risk (Zamora-Ros et al. 2020).

One common limitation among existing studies is the collection of one single biofluid sample. The assessment of multiple biofluid samples, and even combining them with multiple dietary records, should become the gold standard to reflect the habitual intake of flavonoids. Furthermore, there is a need to improve analytical techniques to allow the collection of more data on absorption and excretion of various types of flavonoids.

Dietary flavonoid intake might also be related to several mental health outcomes, including depression (Chang et al. 2016), sleep disorders (Hostetler et al. 2017), and cognitive function (Devore et al. 2012). A Spanish study reported a decline in depression symptoms after an intervention with cocoa supplementation within a hypocaloric diet (Ibero-Baraibar et al. 2015), while urinary flavonoid metabolites (2,5,7,3′,4′-pentahydroxyflavanone-5-*O*-glucoside and 7,4′-dimethoxy-6-C-methylflavanone) were more excreted in the cocoa-supplemented group (Ibero-Baraibar et al. 2016). Data from a cross-sectional study comprising 4830 adults showed an inverse relation between urinary *O*-desmethylangolensin and genistein concentrations, and sleep disorders in women aged 60 years or over (Sun et al. 2020). Although the underlying mechanisms of the association between phytoestrogens and the sleep cycle remain unclear, it has been suggested that their estrogenic or antiestrogenic properties may be a potential explanation.

Overall, studies investigating diet-disease associations using blood and urinary flavonoid biomarkers are limited. Based on the current evidence, a putative inverse association of urinary and blood isoflavones with breast cancer risk and CVD can be concluded. Further investigation in the use of flavonoids for improving cognitive function and mental health is needed.

Dietary Assessment

A great application of blood and urinary flavonoids is their use as biomarkers of both their own dietary exposure and the intake of flavonoid-rich foods. Biomarkers may be particularly useful in nutrition research when reliable food content values are missing and to overcome the measurement errors of self-reported dietary intakes. Previous studies were mostly focused on flavonols and isoflavones and were developed in Asian countries and the USA (Zamora-Ros et al. 2014). Later, this list was extended into three substudies of the European Prospective Investigation into Cancer and Nutrition (EPIC) study (Tahiri et al. 2020; Garro-Aguilar et al. 2020; Almanza-Aguilera et al. 2021) and one Iranian study (Sadeghi et al. 2021) that analyzed the correlations between urinary and dietary intake of flavonols, flavan-3-ols, flavanones, and their main food sources. Table 2 summarizes several studies presenting these correlations. Overall, blood and urinary isoflavones and flavonols, particularly quercetin, can be considered promising biomarkers of their intake. Isoflavones are among the most studied and certainly the best absorbed flavonoids, showing a high recovery yield (12–37%), high correlation with its exposure (0.67–0.87), and good sensitivity and robustness as intake biomarkers throughout different studies (Pérez-Jiménez et al. 2010). Quercetin also presented a good correlation with fruit, berry, and vegetable intake. Likewise, flavanones were good biomarkers of fruit intake, especially citrus fruits. Urinary flavan-3-ols, (+)-catechin and (−)-epicatechin, also showed moderate correlations with tea, wine, and berry intakes (Table 2). In conclusion, high correlations were observed with isoflavone and flavanone biomarkers, while moderate correlations were found with flavonols and flavan-3-ols.

Mini-Dictionary of Terms

1. **24-h dietary recall**. A dietary assessment tool consisting in a structured interview in which people are asked to indicate all foods and beverages and the quantities consumed within the last 24 h.
2. **Bioavailability**. Refers to the fraction (percentage) of an administered dose of a substance or drug that reaches the systemic circulation or the site of action.
3. **Conjugation (biochemical).** Also known as biotransformation. The biochemical modification of one or more chemical compounds to facilitate their excretion. Common conjugations include the formation of glucuronide and sulfate derivates, among others.
4. **Food frequency questionnaire**. A method to estimate the usual amount and frequency of consumption of a number of foods and beverages over a specific period of time.
5. **Secondary metabolism**. Set of biochemical reactions producing (secondary) metabolites not essential for the growth and survival of the organism, but conferring adaptive functions such as pigments, defense against stressors, signaling mediators, etc.

Table 2 Examples of studies showing the capacity to assess flavonoids in human blood and urine as biomarkers of their habitual dietary intake

Biomarker	Flavonoids and flavonoid-rich foods ingested	*r*	Biospecimen	Analytic technique	N	Country or Study	References
Flavonols							
Quercetin	Quercetin	0.51	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Quercetin	Quercetin	0.31	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Garro-Aguilar et al. (2020)
Quercetin	Vegetables[a]	0.22	Plasma (F)	HPLC	140	Iran	Sadeghi et al. (2021)
	Berries	0.20					
	Fruit	0.23					
	Legumes	0.18					
Quercetin	Quercetin	0.30	Plasma (F)	HPLC	48	Germany	Radtke et al. (2002)
	Kaempferol	0.40					
	Total flavonols	0.36					
Kaempferol	Kaempferol	0.44	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Kaempferol	Kaempferol	0.25	Plasma (F)	HPLC	140	Iran	Sadeghi et al. (2021)
	Vegetables	0.24					
Kaempferol	Kaempferol	0.18	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Garro-Aguilar et al. (2020)
Isorhamnetin	Isorhamnetin	0.33	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Total flavonols	Tea	0.20	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Garro-Aguilar et al. (2020)
	Total flavonols	0.23					
Flavones							
Apigenin	Apigenin	0.42	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Luteolin	Luteolin	0.44	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Apigenin, Luteolin	Apigenin + Luteolin	0.46	Plasma (F)	HPLC	92	China	Cao et al. (2010)
Flavanones							
Naringenin	Hesperetin	0.79	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Tahiri et al. (2020)
	Flavanones	0.96					

(continued)

Table 2 (continued)

Biomarker	Flavonoids and flavonoid-rich foods ingested	*r*	Biospecimen	Analytic technique	N	Country or Study	References
	Fruits	0.14					
	Citrus fruits	0.21					
Naringenin	Naringenin	0.47	Plasma (F)	HPLC	48	Germany	Radtke et al. (2002)
	Hesperetin	0.43					
	Flavanones	0.44					
Hesperetin	Flavanones	0.90	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Tahiri et al. (2020)
	Fruits	0.13					
	Citrus fruits	0.17					
Hesperetin	Hesperetin	0.64	Plasma (F)	HPLC	48	Germany	Radtke et al. (2002)
	Naringenin	0.65					
	Total flavanones	0.44					
Total flavanones	Fruits	0.14	Urine (24-h)	UHPLC-MS/MS	475	EPIC	Tahiri et al. (2020)
	Citrus fruits	0.20					
Flavan-3-ols							
(+)-Catechin	Total flavan-3-ols monomers	0.22	Urine (24-h)	UHPLC-MS/MS	419	EPIC	Almanza-Aguilera et al. (2021)
(−)-Epicatechin		0.25					
(+)-Catechin + (−)-epicatechin		0.28					
(+)-Catechin	Tea	0.13	Urine (24-h)	UHPLC-MS/MS	419	EPIC	Almanza-Aguilera et al. (2021)
(−)-Epicatechin		0.20					
(+)-Catechin + (−)-epicatechin		0.20					
(+)-Catechin	Berries	0.14	Urine (24-h)	UHPLC-MS/MS	419	EPIC	Almanza-Aguilera et al. (2021)
(−)-Epicatechin		0.12					
(+)-Catechin + (−)-epicatechin		0.14					
(+)-Catechin	Wine	0.27	Urine (24-h)	UHPLC-MS/MS	419	EPIC	Almanza-Aguilera et al. (2021)
(+)-Catechin + (−)-epicatechin		0.18					

Isoflavones							
Daidzein	Daidzein	0.64	Urine (spot)	LC-MS	24	Korea	Kim et al. (2010)
	Genistein	0.58					
	Glycitein	0.63					
	Isoflavone	0.61					
Genistein	Daidzein	0.62	Urine (spot)	LC-MS	24	Korea	Kim et al. (2010)
	Genistein	0.62					
	Glycitein	0.69					
	Isoflavone	0.64					
Glycitein	Daidzein	0.59	Urine (spot)	LC-MS	24	Korea	Kim et al. (2010)
	Genistein	0.55					
	Glycitein	0.57					
	Isoflavone	0.59					
Daidzein	Daidzein	0.24	Urine (24-h)	GC-MS	105	USA	Atkinson et al. (2002)
Genistein	Genistein	0.31	Urine (24-h)	GC-MS	105	USA	Atkinson et al. (2002)
Daidzein + O-DMA + equol	Daidzein	0.28	Urine (24-h)	GC-MS	105	USA	Atkinson et al. (2002)
Daidzein + genistein + *O*-DMA + equol	Daidzein + genistein	0.29	Urine (24-h)	GC-MS	105	USA	Atkinson et al. (2002)

[a]Cooked vegetables. Abbreviations: EPIC, European Prospective Investigation into Cancer and Nutrition study; F, fasting; GC-MS, Gas chromatography coupled to mass Spectrometry; HPLC, High-performance liquid chromatography; HPLC-MS/MS, High-performance liquid chromatography coupled to tandem mass spectrometry; LC-MS, Liquid chromatography coupled to mass spectrometry; O-DMA, *O*-desmethylangolensin; r, coefficient of correlation; and UHPLC-MS/MS, Ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry

Key Facts of the Flavonoid Exposure Assessment and Their Use as Biomarkers

- The combined use of self-reported questionnaires and food composition databases is currently the fastest and easiest way to estimate flavonoid intake in large epidemiological studies.
- Determination of flavonoid exposure in human biofluids is crucial in nutritional epidemiology to accurately investigate the associations of flavonoid intake with the risk of chronic diseases.
- To date, liquid and gas chromatography-based methods can be considered standard techniques for the identification and quantification of flavonoids in biological samples.
- Despite their high sensitivity and capacity to estimate the concentration of a large number of flavonoids, the use of these methodologies still faces technical and economic challenges, making their application particularly difficult in large-scale studies.
- Inter- and intravariability of flavonoid concentrations in biofluids should be considered a potential limitation when using them as biomarkers.

Summary Points

- Flavonoids exert numerous beneficial health effects in human, acting at different molecular levels and preventing some chronic diseases.
- Traditional methods to estimate flavonoid intake based on questionnaires and food composition databases are susceptible to bias when estimating the real exposure.
- Measurement of flavonoid and flavonoid metabolites in blood fractions such as plasma and serum, and in urine, is a more accurate way to determine such exposures.
- Liquid and gas chromatography coupled to mass spectrometry are powerful and sensitive technologies able to separate and identify different flavonoid subclasses.
- These and other emergent technologies are of great value in nutrition studies, evaluating the exposure to these compounds and their relationships with diseases.
- Measurement of flavonoids in urine, plasma, and serum has been successfully applied to assess their relationship with the incidence of chronic diseases and their potential as dietary intake biomarkers.

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Uric Acid as a Biomarker in Nutritional Metabolism

7

A Focus on Diabetes

Tomislav Bulum

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Abstract

Uric acid (UA) is a weak acid generated from the purine metabolism. UA exists in serum as urate, the salt of uric acid, and varies significantly with diet. The balance of UA levels is driven by ingestion of purines, endocrine production, and excretion. Ingestion of animal proteins is the most important factor leading to hyperuricemia. Hyperuricemia in humans increases the risk of gout, nephrolithiasis, and other comorbidities related to high UA levels. Recently, hyperuricemia is proposed to be a biomarker in several other renal, metabolic, and cardiovascular disorders and to play an important role in vascular inflammation and atherosclerosis. Clinical management of hyperuricemia includes controlling food intake and therapy with

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uricosuric agent. In clinical practice, targeting lower UA levels may help in reducing the risk of development and harmful effects of nowadays frequent metabolic disorders associated with obesity, metabolic syndrome, and type 2 diabetes mellitus.

Keywords

Uric acid · Hyperuricemia · Purine · Diet · Gout · Nephrolithiasis · Xanthine oxidase · Metabolic syndrome · Type 2 diabetes · Cardiovascular disease · Inflammation · Nutrition

Abbreviations

ATP	Adenosine triphosphate
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
ED	Endothelial dysfunction
IR	Insulin resistance
MS	Metabolic syndrome
MUC	Monosodium urate crystals
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
T2DM	Type 2 diabetes mellitus
UA	Uric acid
XO	Xanthine oxidase

Introduction

Uric acid (UA) is a weak acid generated from the purine metabolism (Griebsch and Zollner 1974). UA then undergoes oxidative degradation with uricase enzyme creating stable allantoin compound (Wu et al. 1989). However, humans cannot oxidize UA to allantoin because in humans uricase gene is crippled by mutations resulting in significantly higher UA levels (up to ten times) compared to others mammals (Wu et al. 1992). UA exists in serum as urate, the salt of uric acid. Increasing urate concentration in blood is associated with formation of uric acid crystal. Serum levels of UA vary significantly with diet, and ingestion of animal proteins is the most important factor leading to hyperuricemia. Increased UA production and/or decreased UA excretion leads to hyperuricemia (Su et al. 2014). Hyperuricemia results in deposition of urate crystals in synovial fluid and tissues increasing the risk of comorbidities related to high UA levels, particularly gout and nephrolithiasis (Wu et al. 2014; Mandal and Mount 2014). UA is produced endogenously mainly in the liver and intestines and eliminated from the body via gastrointestinal tract and kidneys (Roch-Ramel and Guisan 1999; Chaudhary et al. 2013). Although almost all UA is filtered from glomeruli, approximately 90% is then

reabsorbed via proximal tubule back into blood (Chaudhary et al. 2013). In humans with decreased renal function, UA excretion may be impaired causing hyperuricemia (Jin et al. 2012).

Uric Acid Metabolism

UA is generated from the purine metabolism, and purines are the main components of nucleotides. In purine metabolism the final stage includes hypoxanthine catalyzation to xanthine and then to UA by xanthine oxidase (XO) (Fig. 1). Conversion of hypoxanthine and xanthine to UA can be stopped with XO inhibitor drugs like allopurinol. Three urate transporters, URAT1/SLC22A12, GLUT9/SLC2A9, and ABCG2/BCRP, are involved in the homeostasis of serum UA level. Among them, dysfunction of ABCG2 exporter has been confirmed as a major cause of hyperuricemia and gout (Matsuo et al. 2014). ABCG2 is an adenosine triphosphate (ATP)-driven efflux pump, and genetic variation in ABCG2 would lead to increased urate reabsorption via kidneys and reduced ABCG2-mediated urate secretion via kidneys that represent the major underlying mechanism leading to the hyperuricemia in patients with gout (Mandal and Mount 2014). The URAT1 protein, encoded by the SLC22A12 gene, is present in the human proximal tubule and implicated in the physiology of urate homeostasis (Maiuolo et al. 2013). Finally, GLUT9 (SLC2A9) is a membrane transporter and compared to others of the GLUT superfamily that transport glucose or other monosaccharides, GLUT9 transport urate in the main organs involved in urate transport, particularly in the liver and kidney (Clémençon et al. 2014).

High intake of food and products rich in UA is the main cause of hyperuricemia (Table 1). Fructose is a unique sugar molecule that increases the amounts of UA. Hyperuricemia results in deposition of urate crystals in synovial fluid and tissues leading to development of gout, the traditional medical condition associated with hyperuricemia, clinically presented by hot, red, and swollen joints that finally after recurrent attacks can cause acute inflammatory arthritis (Albrecht et al. 2014). However, hyperuricemia can be protective in the presence of elevated oxidative

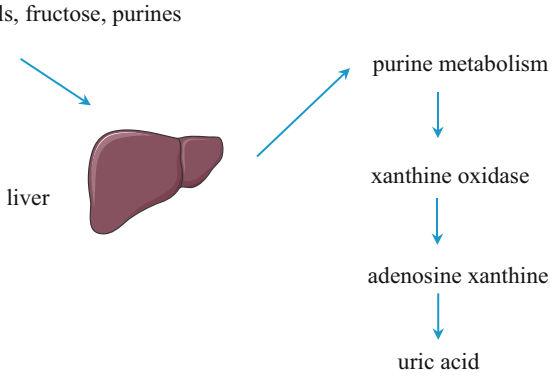

Fig. 1 Uric acid metabolism

Table 1 High and low purine food and products intake

High purine food and products	Low purine food and products
Meats (particularly brains, kidneys, liver, mincemeat) Beer and alcoholic drinks Seafood (particularly anchovies, sardines, mackerel, herring, sardines, mussels) Vegetables: asparagus, cauliflower, kidney beans, lentils, lima beans, mushrooms, navy beans, peas, spinach Yeast and yeast extracts	Dairy products: milk, butter/margarine, cheese Eggs Cereals/cereal products Fruits and vegetables (except those listed in high purine food) Gelatine Water, coffee, tea, juices, cocoa Polyphenols and flavonoids L-Glutamine and L-arginine Vitamin C and herbals

stress (Ames et al. 1981). UA has both an antioxidant effect and prooxidant effect on lipids (Patterson et al. 2003). Acute increase of plasma UA increases plasma antioxidant capacity and reduces oxidative stress in humans and reduces oxidative damage related to aging atherosclerosis (Vukovic et al. 2009). Hyperuricemia also has a protective effect in some neurodegenerative diseases like Alzheimer's disease/dementia, Parkinson's disease, and multiple sclerosis (Clémençon et al. 2014). However, although hyperuricemia has both inflammatory and oxidative/anti-oxidative effects in various organs, its negative effects appear to outweigh the beneficial effects in most cases.

Inflammation and Uric Acid Metabolism

Gout is the most common and traditional medical condition related to hyperuricemia. Chronic inflammation associated with UA metabolism is underlying conditions that cause gout development and organ dysfunction. Several metabolites, such as monosodium urate crystals (MUC) or ATP, are implicated in the activation of inflammasomes. Inflammasomes play a role in immune responses against different pathogens and involve multiple proteins (Schroder and Tschopp 2010; Medzhitov 2008). Activation of inflammasomes via MUC and their inflammatory responses are implicated in in the onset and progression of different diseases including gout. Besides inflammasomes, superoxide free radicals generated by XO also play physiological roles in different diseases. XO activity is rich in human microvascular endothelial cells and macrophages leading to endothelial dysfunction (ED) (Spiekermann et al. 2003). XO activity is also elevated with aging and hypoxia-associated conditions (Nicholas et al. 2011; Aranda et al. 2007). Hyperglycemia also increases XO activity (Malardé et al. 2014). Superoxides produced by XO induce inflammation and tissue damage. XO induces inflammatory cytokines and lipid deposition in cells and also appears to be critical for innate immune function (Kushiyama et al. 2012; Vorbach et al. 2003). Taken together, superoxide from XO is involved in various inflammatory and/or ischemic pathophysiology processes including hyperuricemia.

Uric Acid Metabolism and Metabolic Disorders

Besides gout, the best known disease associated with hyperuricemia, several other metabolic disorders are linked to hyperuricemia (Fig. 2). Relationship between UA and chronic kidney disease (CKD) is also well known. Lower kidney function results in lower excretion of UA via kidneys leading to hyperuricemia. However, hyperuricemia can be both a cause and a consequence of CKD. Hyperuricemia activate the renin-angiotensin system implicated in the development of CKD leading to vascular smooth muscle cell proliferation (Kosugi et al. 2009). In addition, XO simultaneously produce UA and superoxides that induces inflammation and tissue damage implicated in pathophysiology of CKD (Ohtsubo et al. 2004). Hyperuricemia is also a risk factor for development of hypertension and cardiovascular disease (CVD), hyperuricemia increases risk of CVD morbidity and mortality, and therapy with uricosuric drug reduces blood pressure (Sundström et al. 2005; Agarwal et al. 2013; Li et al. 2016). Underlying condition that connects hyperuricemia and CVD includes XO which is expressed in endothelial cells and induces inflammation and tissue damage (Nomura et al. 2014). In addition, XO activity in macrophages increases the uptake of lipids which represent a key role in the development of atherosclerosis (Kushiyama et al. 2012). Endothelial cells also express several UA transporters, and hyperuricemia has direct effects on ED via impairing nitric oxide production (Mishima et al. 2016).

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disorder, with a high prevalence worldwide of 25%. Patients with NAFLD have significantly higher UA levels than those without NAFLD (Li et al. 2009). Serum UA is assumed to be a risk factor for the development and/or progression of NAFLD (Sertoglu et al. 2014). Hyperuricemia increases fat depositions and intracellular triglycerides contents in hepatocytes which represent the major pathophysiological process leading to NAFLD (Lanaspa et al. 2012a). UA and superoxides simultaneously produced by XO also induce endoplasmic reticulum stress (Choi et al. 2014). In addition,

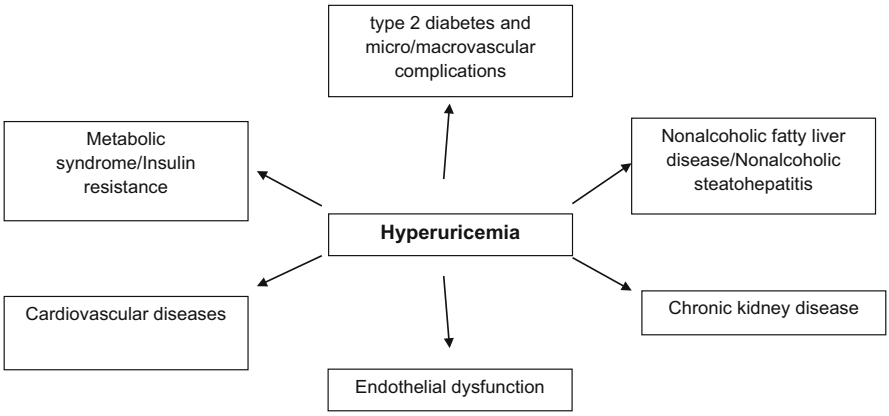

Fig. 2 Hyperuricemia and metabolic disorders

inflammasomes activated with UA are key players in development and progression of nonalcoholic steatohepatitis (NASH), the heavier form of NAFLD (Henao-Mejia et al. 2012). Hyperuricemia is also related to insulin resistance (IR), the underlying condition connecting obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus (T2DM) (Tae et al. 2005). In patients with metabolic syndrome (MS) and IR, treatment with uricosuric agent decreases oxidative stress and improves endothelial function (Yiginer et al. 2008). High fructose intake is one of the major causes of the development of IR and MS. UA is involved in fructose metabolism and promotes fat deposition and glucose production (Lanaspa et al. 2012b). UA induces oxidative stress in cells and induces de novo lipogenesis that is implicated in the development of IR and T2DM (Zhu et al. 2014).

Nutritional Approach to Hyperuricemia

Endogenous UA arises from the de novo synthesis and catabolism of nucleic acids. UA derives also from the metabolism of purine compounds in foods in the intestine. Traditional high purine-containing foods include all meats, seafood, vegetables (peas, beans, lentils, asparagus, spinach, and mushrooms), yeast and yeast extracts, and beverages (beer and alcoholic drinks) (Fam 2002) (Table 1). UA is produced by the liver metabolism of fructose. Fructose occurs in fruits and vegetables, and the major fructose source is fruits, fruit juices, honey, and sweeteners sucrose, particularly white sugar. In nature, fructose occurs as a single molecule (monosaccharide) or bound to a glucose molecule in the disaccharide sucrose (Muir et al. 2007). Fructose is present in up to 50% of the artificial sweeteners. Fructose ingestion is associated with the acute increases in UA regardless of the source (White et al. 2018). In addition, the metabolism of fructose, compared to the dietary purines, produces additional toxins besides UA inducing oxidative stress and proinflammatory effects on a wide range of tissues (Zhang et al. 2017). Fructose metabolism is also associated with de novo lipogenesis and hepatic fat accumulation followed by an acute rise in UA levels.

Common table sugar sucrose is a disaccharide composed of fructose and glucose. Over the last decades, sugar intake in developed countries has increased dramatically. Glucose is absorbed in the small intestine, and then most of them in free form enters the portal vein and dissolves into the blood. Glucose is the strongest stimulus that induces insulin secretion and then entered into cells. However, in excessive consummation glucose is stored in the liver and muscles increasing IR. Compared to glucose, only small amounts of fructose are entering in the blood, and fructose is metabolized differently independently of insulin by the liver (McMullen 2018). Consequently, fructose has a low glycemic index.

Clinical management of hyperuricemia includes controlling food intake and therapy with uricosuric agent. Dietary interventions include limiting the intake of fructose-rich and purine-rich foods. Traditional list of low purine-containing foods includes dairy foods (milk, cheese, butter, eggs), grains/cereals (bread, pasta, cakes), vegetables (fruits and nuts (except beans, lentils, asparagus, spinach)), sugar (sweets

and gelatine), and beverages (water, coffee, cocoa, tea, juices, and carbonated beverages) (Fam 2002). The Dietary Approaches to Stop Hypertension (DASH) diet is also suitable and recommended to control hyperuricemia (Sacks et al. 2001). Although DASH diet is designed to lower blood pressure, this diet involves low salt intake; high dairy, fruit, grain, and vegetable intake; and the limitation of fish/meat and also lowers UA and risk of IR (Liese et al. 2009). Hyperuricemia is associated with alcohol intakes, particularly beer, but not with wine drinking (Choi et al. 2004).

Diets rich in polyphenols, and particularly flavonoids, also decrease UA levels and play a role in the prevention of T2DM (Knaze et al. 2018). Polyphenols decrease UA levels and consequently decrease arginase activity and restore ED in a dose of 500 mg daily (Minozzo et al. 2018). Amino acid L-glutamine lowers UA via increasing renal glomerular filtration rate (Welbourne et al. 1998). L-Glutamine also induces secretion of UA in the digestive juices because L-glutamine is the major fuel for the enterocytes (Brasse-Lagnel et al. 2010). Similar to L-glutamine, L-arginine facilitates intestine cell proliferation and inhibits inflammation and apoptosis. L-Glutamine and L-arginine are well tolerated and safe in small doses up to 20 g/day (Shao and Hathcock 2008). An increased intake of magnesium and citrate has beneficial effects on hyperuricemia and particularly on risk of kidney stones (Zhang and Qiu 2018). Vitamin C is a well-known antioxidant and also decreases UA via renal excretion in a dose of 500 mg daily (Juraschek et al. 2011).

Herbals are also used to treat hyperuricemia and act as diuretics and by increasing renal excretion rate decrease level of UA. Although traditional herbal treatments for hyperuricemia are used a long period of time, only treatment with cranberry has been evidence-based recognized (Braun and Cohen 2015). Cranberry juice decreases urinary pH and increases urate excretion via kidneys (Keßler et al. 2002). Ingestion of 1000 mg curcumin daily decreases UA inhibiting XO and URAT1 urate transporter (Ao et al. 2017). Finally, the well-known and often used antioxidant green tea inhibits XO activity and reduces UA (Jatuworapruk et al. 2014).

Uric Acid and Diabetes

IR is an underlying condition for development of MS and T2DM. Patients with T2DM have higher levels of UA compared to healthy subjects because hyperuricemia is linked to hyperinsulinemia and IR and also considered being one of the components of MS (Bo et al. 2001). High UA levels significantly correlate with individual components of MS (abdominal obesity, hypertension, hyperglycemia, and dyslipidemia) and predict MS (Yoo et al. 2005). Since hyperuricemia is associated with IR and MS and with individual components of MS, it is also involved in the development and progression of micro- and macrovascular complications of diabetes (Kushiyama et al. 2014; Xiong et al. 2019) (Fig. 3). Diabetic neuropathy is often the initial manifestation of T2DM, and UA levels are increased in patients with neuropathy even after controlling for traditional confounding factors (Kiani et al. 2014). Possible relationship between UA and diabetic neuropathy is probably related

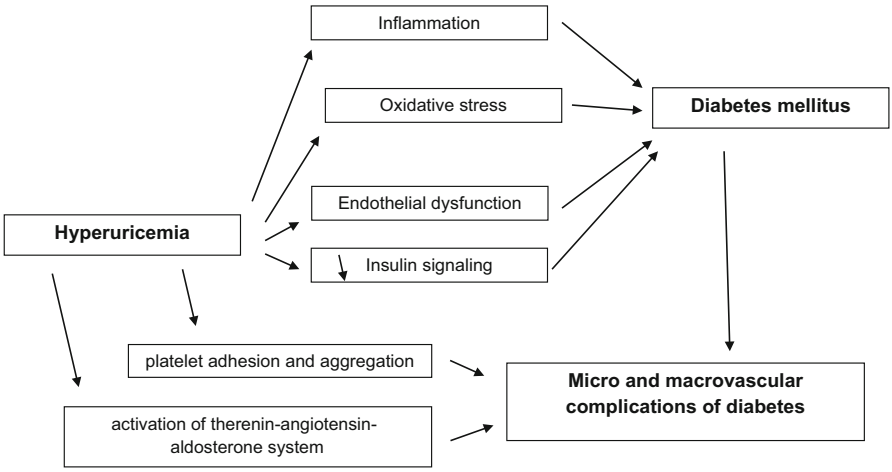

Fig. 3 Uric acid and diabetes mellitus

with oxidative stress and inflammation associated with XO activity. Higher UA levels are also connected with a higher prevalence and increased severity of diabetic retinopathy. UA levels are higher in vitreous in patients with proliferative compared to those with nonproliferative diabetic retinopathy, and focal UA production in the vitreous is implicated in the pathogenesis and progression of diabetic retinopathy (Krizova et al. 2011).

High UA levels are associated with CKD development and progression and with known risk factors associated with CKD. UA is associated with CKD development and progression even in patients with initially preserved kidney function and high-normal serum UA, independently of glucose control (Kim et al. 2014; Tanaka et al. 2014). Higher UA levels is a risk factor for accelerated decreases of renal function suggesting that higher production of UA is implicated in the pathophysiology of CKD (Tanaka et al. 2014). In addition, in hyperuricemic patients with CKD, treatment with XO inhibitor preserves kidney function (Siu et al. 2006). Treatment with XO inhibitor allopurinol also suppresses growth factor activation in tubular cells in an animal model with diabetic nephropathy (Kim et al. 2012). Higher UA levels are also a significant and independent risk factor for diabetic foot ulcer, another diabetic complication comprised of both micro- and macrovascular changes (Ye et al. 2014).

UA is an independent risk factor for macrovascular complications in T2DM, even after controlling for renal function (Tanaka et al. 2011). Higher UA levels are associated with carotid atherosclerosis and increase risk of fatal stroke (Gerber et al. 2006). Treatment with XO inhibitor allopurinol has protective effects in patients with chronic stable angina and protects the heart from ischemic reperfusion (Noman et al. 2010). Treatment with allopurinol also improves the left ventricular ejection fraction in patients with congestive heart failure (Cingolani et al. 2006). XO inhibitors reverse ED in smokers and improve endothelium-dependent vascular

relaxation in animal models (Guthikonda et al. 2003). XO inhibitors through inhibition of XO activity reduce migration of neutrophils and chemoattractant secretion from mononuclear phagocytes preventing the development of atherosclerosis (Gibbings et al. 2011). Deposition of UA is seen in macrophages in arteriosclerotic lesions, and treatment with XO inhibitor inhibits foam cell formation in macrophage improving calcification and lipid accumulation in atherosclerotic plaque (Nomura et al. 2014). XO also induces cyclooxygenase to induce inflammation. Treatment with allopurinol inhibits secretion of various inflammatory cytokines (Kushiyama et al. 2012). In addition, accumulation of UA crystal in atherosclerosis lesion might activate inflammation in inflammatory cells (Kushiyama et al. 2014). Since rates of T2DM and obesity are increasing rapidly worldwide, underlying metabolic disorders that include IR, MS, fat deposition, chronic inflammation, ED, and oxidative stress are closely associated with metabolism of UA playing an important role in the pathogenesis of T2DM and its complications.

Conclusion

Elevated serum levels of UA have been shown to be a biomarker in a wide range of metabolic, renal, and cardiovascular disorders and to play an important role in vascular inflammation and atherosclerosis. The balance of UA levels is driven by ingestion of purines, endocrine production, and excretion. In case of hyperuricemia, diet is recommended along with treatment with XO inhibitors. High UA levels affect IR and predict MS and significantly correlate with individual components of MS (abdominal obesity, hypertension, hyperglycemia, and dyslipidemia) leading to development of T2DM and its complications. In clinical practice, targeting lower UA levels may help in reducing the risk of development and harmful effects of nowadays frequent metabolic disorders associated with obesity, MS, and T2DM.

Applications to Prognosis and Other Diseases or Conditions

In this study we review a uric acid as a biomarker in nutritional metabolism focusing on diabetes. Uric acid metabolism has both inflammatory and oxidative/antioxidative effects in many organs, but its negative effects appear to outweigh the beneficial effects in most cases (Mandal and Mount 2014; *Patterson et al.* 2003*). Hyperuricemia in humans increases the risk of gout, nephrolithiasis, and other comorbidities related to high UA levels. Recently, hyperuricemia is proposed to be a biomarker in a several other renal, metabolic, and cardiovascular disorders associated with metabolic syndrome and insulin resistance like type 2 diabetes and its complications and to play an important role in vascular inflammation and atherosclerosis (Kushiyama* et al. 2014*, 2016). Clinical management of hyperuricemia includes controlling food intake and therapy with uricosuric agent (Mcmullen* 2018*). In clinical practice, targeting lower UA levels may help in treatment not only traditional condition associated with hyperuricemia like gout and nephrolithiasis*

but also to reduce the risk of development and harmful effects of nowadays frequent metabolic disorders associated with obesity, metabolic syndrome, and type 2 diabetes.

Mini-Dictionary of Terms

- ***Uric acid**. Weak acid generated from the purine metabolism. Uric acid exists in serum as urate, the salt of uric acid.*
- ***Insulin resistance**. Underlying condition for development of metabolic syndrome and type 2 diabetes. It is characterized with abdominal obesity.*
- ***Metabolic syndrome**. Cluster of disorders associated with insulin resistance and obesity. It includes hyperglycemia, dyslipidemia, hypertension, and abdominal obesity.*
- ***Xanthine oxidase (XO) inhibitors**. Drugs that prevent the conversion of hypoxanthine and xanthine to uric acid. They are widely used to treat patients with both symptomatic and asymptomatic hyperuricemia.*
- ***Gout**. The best known disease associated with hyperuricemia. It is characterized with deposition of urate crystals in the joints and soft tissues, joint swelling, and articular and periarticular inflammation and injury.*

Key Facts of Uric Acid

A weak acid generated from the purine metabolism.

Ingestion of animal proteins is the most important factor leading to high levels of uric acid.

High levels of uric acid in humans increase the risk of gout, nephrolithiasis, and other comorbidities.

Nowadays, elevated serum levels of uric acid have been shown to be a biomarker in a wide range of other metabolic, renal, and cardiovascular disorders and to play an important role in vascular inflammation and atherosclerosis.

Clinical management of high uric acid level includes controlling food intake and therapy with uricosuric agent.

Summary Points

- *Uric acid is a weak acid generated from the purine metabolism.*
- *Uric acid exists in serum as urate, the salt of uric acid.*
- *Serum levels of uric acid vary significantly with diet, and ingestion of animal proteins is the most important factor leading to hyperuricemia.*
- *Hyperuricemia in humans causes deposition of urate crystals in the joints and kidneys increasing the risk of gout, nephrolithiasis, and other comorbidities.*

- *Nowadays, elevated serum levels of uric acid have been shown to be a biomarker in a wide range of other metabolic, renal, and cardiovascular disorders and to play an important role in vascular inflammation and atherosclerosis.*
- *Clinical management of hyperuricemia includes controlling food intake and therapy with uricosuric agent.*
- *Targeting lower UA levels may help in treatment not only traditional condition associated with hyperuricemia like gout and nephrolithiasis but also to reduce the risk of development and harmful effects of nowadays frequent metabolic disorders associated with obesity, metabolic syndrome, and type 2 diabetes.*

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Ceramides as Dietary Biomarkers

8

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Abstract

Ceramides are components of all cell membranes, regulating immune cell function and inflammation. Metabolism-wise, ceramides increase fat use as an energy substrate instead of glucose, thus reducing insulin resistance. In parallel, mitochondrial performance is also reduced, enabling the consumption of more fat per unit of energy production, while disrupting the function of lipolysis enzymes. Increased ceramide concentrations are observed in the adipose tissue of individuals with obesity, while in mice, blocking ceramide production improved insulin sensitivity and prevented atherosclerosis and heart failure. Elevated ceramide concentrations are associated with adiposity and the development of insulin resistance, type 2 diabetes mellitus, and increased cardiovascular disease risk. Interventions with hypocaloric diets, adherence to the Mediterranean diet, low-sugar intake, substitution of unhealthy dietary fats, and supplementation with n-3 fatty acids and vitamin D can reduce ceramide concentrations, improving prognosis.

Keywords

Lipidome · Lipidomics · Metabolome · Oral nutrient supplementation · Metabolic inflexibility · CVD risk · Metabolic risk · Adiposity · Type 2 diabetes mellitus · Insulin resistance

Abbreviations

C1P	Ceramide 1-phosphate
CAD	Coronary artery disease
CerS	Ceramide synthetase
CVD	Cardiovascular disease
EVOO	Extra-virgin olive oil
FFA	Free fatty acids
GLUT-4	Glucose transporter type *4*
HELLP	Hemolysis, elevated liver enzymes, and low platelets
HOMA-IR	Homeostatic model assessment of insulin resistance
IR	Insulin resistance
MD	Mediterranean diet
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
OA	Oleic acid
PA	Palmitic acid
PC	Phosphatidylcholine
PKB	Akt kinase/protein B
PREDIMED	PREvención con DIeta MEDiterránea
RCT	Randomized controlled trial

S1P	Sphingosine 1-posphate
SFA	Saturated fatty acid
SPT	Serine palmitoyl-transferase
T2DM	Type 2 diabetes mellitus
TAG	Triacylglyceride
TNFα	Tumor necrosis factor-α
VD	Lacto-ovo-vegetarian diet

Introduction

The sphingolipid family consists of one of the major lipid classes, including the glycosphingolipids (like gangliosides) and the sphingomyelins (which are also phospholipids) (Gault et al. 2010). They are components of all cell membranes regulating the interaction and recognition between cells and the introduction of pathogens (Hannun and Luberto 2004).

Ceramides are precursors of sphingolipids and are characterized by a major amino alcohol group, consisting of usually 18 carbon atoms, sphingosine, to which amide-bonded fatty acid molecules are attached (Merrill 2002). Because of the wide heterogeneity in the number of fatty acid carbons, the position, and the degree of saturation and hydroxylation, ceramides are characterized as a group of compounds rather than individual species. In mammals, although the length of the aliphatic chains varies between 14 and 36 carbons, the most common tissue ceramides have up to 24 atoms (Norris and Blesso 2017).

Ceramides are biologically active mainly in transmembrane signaling, playing an important role in regulating immune cell function, lipotoxicity, and inflammation (Haus et al. 2009). Ceramides are also the second major messengers of the inflammatory response induced by the tumor necrosis factor-α (TNFα). Apoptosis and differentiation inducers, damaging agents, and inflammatory cytokines seem to increase ceramide production (Górska et al. 2002).

Ceramides are important bioactive effectors of cellular metabolism, regulating the production of various sphingolipids, including glucosylceramides and sphingomyelins. In parallel, they serve as signaling molecules during apoptosis (tumor-suppressive action) and cell growth and differentiation, producing molecules such as sphingosine, sphingosine 1-phosphate (S1P), and ceramide 1-phosphate (C1P) (Hannun and Luberto 2004). Overall, S1P and ceramides are considered as sphingolipid mediators, with their opposing signaling pathway suggested to act as a "sphingolipid rheostat" (Cuvillier et al. 1996; Moro et al. 2019). The ceramide metabolic pathway is activated when the cell does not have an immediate need for lipids and the body stores are full, so that additional lipid molecules will not modify the membranes' structure (Summers et al. 2019). Ceramides manage the cell's extra fatty acids by facilitating their conversion to acyl-CoA and activating their storage-inducing genes. Moreover, they increase fat use as an energy source instead of glucose, thus reducing insulin resistance (IR). In parallel, mitochondrial performance is reduced, enabling the consumption of more

fat per unit of energy production while disrupting the function of lipolysis enzymes (Summers et al. 2019).

Regarding the nomenclature of ceramides, it includes the number of hydroxyl groups, the carbon atoms and double bonds of sphingosine, and the carbon atoms of the aliphatic chain. For example, CER d18:1/24:0 is a ceramide with sphingosine, two hydroxyls (as for "d"), one double bond, and a 24-carbon atom acyl chain (Norris and Blesso 2017).

Following the Metabolic Pathway of Ceramides

Ceramides are produced in the body through three main paths (Fig. 1). The first is de novo, taking place inside the endoplasmic reticulum. It is proceeded by the concentration of palmitoyl-CoA with serine and, after two reactions, the production of sphinganine. The first reaction is catalyzed by the enzyme serine palmitoyl-transferase (SPT). This step is a set point in the synthesis of ceramides (Merrill 2002). The second reaction is performed by the enzyme 3-ketosphinganine reductase (Gault et al. 2010). Subsequently, by fatty acylation, sphinganine is converted to dihydroceramide by the enzyme ceramide synthetase (CerS). Involvement of the dihydroceramide desaturase dihydroceramide enzyme, in turn, will produce a ceramide (Hannun and Obeid 2018). In yeast, there are dihydroceramides and phytoceramides, coming from adding acyl chains to sphinganine (dihydrosphingosine) and phytosphingosine, respectively (Nakahara et al. 2012).

Ceramide synthetase (CerS) is a pivotal enzyme for ceramide formation. There is a group of six distinct CerS enzymes, which are located on five different chromosomes and are expressed in different tissues in humans. These enzymes catalyze the

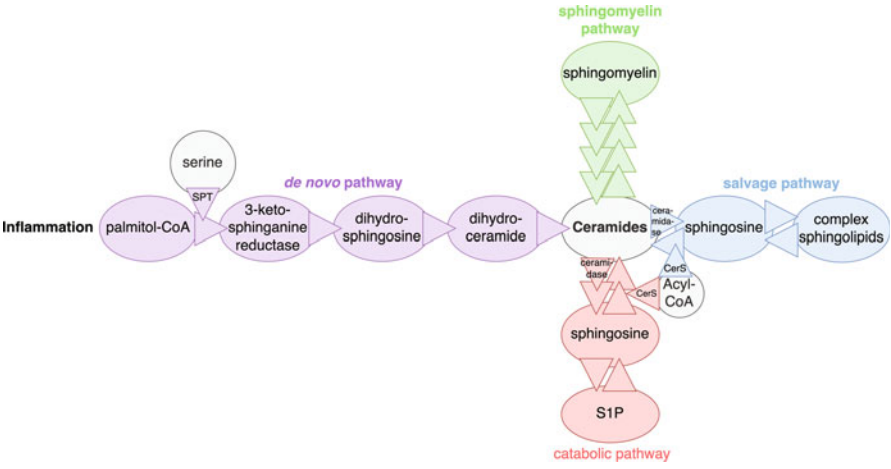

Fig. 1 Ceramide synthesis pathways. *S1P* sphingosine 1-posphate. *SPT* serine palmitoyl-transferase

transport of fatty acyl-CoA with specific carbon atom numbers and saturation degrees (Pewzner-Jung et al. 2006). In the reactions cycle, ceramides can be converted to the more complex glycosphingolipids, C1P, and sphingomyelins, with the help of ceramide and sphingomyelin synthetases (Hannun and Obeid 2018). In the case of sphingomyelin, fatty acids are usually derived from phosphatidylcholine (PC) (Gault et al. 2010).

The second route of ceramide synthesis involves two-way reactions, where sphingomyelin, glycosphingolipids, and C1P are hydrolyzed by sphingomyelinases, acid β-glucosylceramidases, and C1P phosphatases to ceramides (Kihara et al. 2007; Hannun and Obeid 2018) (Fig. 1). The choice of composition path is determined by the type of tissue and/or the type of stimulus it receives (Pewzner-Jung et al. 2006).

The third route of ceramide synthesis is the "salvage pathway" (Chaurasia et al. 2020) (Fig. 1). This route involves the reformation of ceramides from sphingolipids after they are degraded in late lysosomes or endosomes (Kitatani et al. 2008), liberating a sphingoid base that is re-acylated to resynthesize ceramides.

Catabolism of ceramides into sphingosine takes place in the presence of ceramidase enzymes, in a two-way reaction (Fig. 1). Then, S1P can be produced under the action of a kinase (Kihara et al. 2007). Ceramide catabolism in their basic components is mediated by an enzyme group, the ceramidases, divided into acidic, basic, and neutral according to their optimum operating pH. They are all detected in various tissues, except the neutral one, which has an active role in the catabolism of food sphingolipids and is detected in the intestine; it also appears to be involved in various metabolic and degenerative diseases (Parveen et al. 2019). As mentioned above, the reaction is bidirectional, and sphingosine with fatty acylation via CerS enzyme may again yield ceramide if found in abundance from an external or internal source (Merrill 2002). Eventually, sphingolipid catabolism leads to a common pathway, where via two-way reactions, sphinganine and S1P lead to the formation of palmitoyl-CoA. Specifically, they are cleaved by a lyase to a fatty aldehyde, hexadecenal, which can be formed into palmitoyl-CoA (Nakahara et al. 2012).

Recently, substrates such as alanine, glycine, and stearate have been associated with the metabolism of sphingolipids and can be managed by key enzymes such as the SPT. In this manner, the heterogeneity of the ceramides is further increased, as many different sphingoid backbones are created, which are joined with different acyl chains (Hannun and Obeid 2018).

Ceramides: Metabolic Messengers Driving Cardiovascular Diseases (CVDs)

Increased ceramide concentrations are observed in the adipose tissue of individuals with obesity, while in mice, blocking ceramide production improved insulin sensitivity and prevented atherosclerosis and heart failure. A possible mechanism explaining this observation involves the circulation of ceramides inside the adipocytes. Ceramides block the glucose transporter type 4 (GLUT-4) translocation, thus inhibiting glucose uptake in cultured adipocytes (Li et al. 2020b).

In the liver and skeletal muscle, they seem to antagonize insulin signaling. The role of ceramides and other lipids in situations of lipotoxicity seems to be independent of dietary intake. A dominant theory describes ceramides as signals for free fatty acids (FFAs) to protect the cell by neutralization into the triacylglyceride (TAG) pools (Lair et al. 2020).

Evolutionary mechanisms equipped human cells with compounds enabling excess FFAs buffering into macromolecules, ensuring that intracellular levels remain in control (Summers et al. 2019). Thus, different ceramide species are accumulated in the circulation and hepatic and extrahepatic tissues (the muscle and adipose tissue) as a product of local synthesis due to excess in either saturated or unsaturated fatty acids (Sokolowska and Blachnio-Zabielska 2019; Öörni et al. 2020). As a result, muscle ceramide content is greatly related to the plasma FFA concentration, with elevated intramyocellular lipid concentrations being known contributors to the development of IR (Adams et al. 2004). Weight gain and obesity are associated with an increased intramyocellular ceramide content, and this, in turn, appears to reduce the ability of insulin to stimulate glucose uptake in the skeletal muscle of patients with obesity (Adams et al. 2004).

As ceramides accrue, they trigger protective actions towards the cell's increases in FFA content (including altering the cellular substrate preference to fat and enhancing triglyceride storage) (Summers 2020). Moreover, increases in the plasma FFA concentrations multiply the rate of ceramide synthesis and muscle ceramide content (Zabielski et al. 2017). In prolonged accumulative FFA and ceramide concentrations, IR is initiated, parallel to hepatic steatosis, driving the development of cardiometabolic diseases (Summers 2020). The C16:0 ceramide, in particular, is considered the principal mediator of impaired FFA oxidation interfering with glucose uptake, promoting the development of obesity-driven hepatic IR and hepatic steatosis (Kolak et al. 2007; Fucho et al. 2017). CerS6, the enzyme responsible for synthesizing C16:0, is also elevated in the adipose tissue of obese individuals (Li et al. 2020b). By blocking the activation of the anabolic enzyme Akt kinase/protein B (PKB) and simultaneously inhibiting the transmission of signals through the phosphatidylinositol-3 kinase (PI3K), ceramides appear to act antagonistically against insulin signaling (Powell et al. 2003). Nevertheless, the accumulation of FFA is not always positively related to the tissue's sensitivity to uptake insulin (Sokolowska and Blachnio-Zabielska 2019). Furthermore, according to recent research, the skeletal muscle does not export ceramides into the circulation, as reductions in C18:0 ceramide concentrations in the skeletal muscle do not always coincide with changes in circulating C18:0 ceramide levels (Turpin-Nolan et al. 2019).

Many theories support the link between ceramides and inflammatory-related disorders, including obesity. Within the adipose tissue, the generation of ceramides due to sphingomyelinase action appears to promote inflammation. Thus, irrespective of obesity status, those with increased intra-abdominal sphingomyelinase mRNA content appear to be more inflamed compared with those exhibiting lower sphingomyelinase mRNA concentrations (Kolak et al. 2012).

The Dallas Heart Study was the first large-scale cohort (including 1557 participants) revealing a positive correlation between shorter-chain saturated fatty acid

(SFA) ceramides (C18, C16, C20) with adiposity, lipid, and IR. However, plasma ceramide concentrations were not independently associated with type 2 diabetes mellitus (T2DM) after adjustment for clinical factors (Neeland et al. 2018). A meta-analysis pooling 2337 participants from the Strong Heart Study and the Strong Heart Family Study reported elevated plasma ceramide (C18, C20, C22) concentrations in patients who developed T2DM later on in life. Possible explanatory mechanisms for this phenomenon include the fact that ceramides inhibit insulin intermediates, such as the insulin receptor substrates, Akt and GLUT-4, promoting β-cell apoptosis and dysfunction (Fretts et al. 2020). Additionally, a correlation was noted between elevated plasma ceramide concentrations and higher homeostatic model assessment of IR (HOMA-IR), indicating the existence of an association between plasma ceramide concentrations and IR severity (Lemaitre et al. 2018). Based on these findings, ceramides are now considered a promising biomarker for T2DM and a possible therapeutic target (Raichur et al. 2019).

Several lines of evidence suggested that in T2DM, plasma ceramide concentrations are increased (Watt et al. 2012), and the same observation has been reported for patients with a metabolic syndrome diagnosis (Choromańska et al. 2019). Different species appear to act as "metabolic messengers" (Summers et al. 2019), with their synthesis being propelled by any underlying metabolic disease. More recently, preclinical studies revealed that when acid ceramidase-1 is depleted, this leads to ceramide accumulation, reduced energy expenditure, and exacerbation of IR and body weight gain (Chaurasia et al. 2021). On the other hand, research on rodents showed that inhibition of ceramide synthesis induces browning in the white adipose tissue, enabling thermogenesis while reducing systemic inflammation (Chaurasia et al. 2016). Thus, blocking ceramide synthesis within the thermogenic adipocytes could consist of a novel therapeutic pathway to improve adipose health (Chaurasia et al. 2021).

Research in mice (Kasumov et al. 2015) examined the role of ceramides in non-alcoholic fatty liver disease (NAFLD) and associated atherosclerosis. Dietary fatty acid and cholesterol intake are both associated with the circulating ceramide concentrations and their harmful role in the development of atherosclerosis. Pharmacological regression of ceramide biosynthesis with myriocin reduced cholesterol absorption (Kasumov et al. 2015). Other animal studies aimed at inhibiting ceramide biosynthesis from improving IR, liver steatosis, and CVD factors. One study tampered down C16:0 production targeting CerS6 in mice, presenting similar findings (Raichur et al. 2019). Additionally, a negative correlation was observed between ceramides and adiponectin concentrations and a positive correlation between ceramides and TNFα in fat tissues, emphasizing the role of ceramides in the development of metabolic syndrome (Blachnio-Zabielska et al. 2018). These findings are promising, but further research is required to explain the complicated mechanisms of lipid metabolism.

With obesity and CVD having a tight direct association, studies on Caucasian and Chinese cohorts suggest that elevated concentrations of C16:0, C18:0, C24:1 ceramides and low levels of C24:0 are associated with an increased incidence of future cardiovascular events and cardiovascular mortality (Laaksonen et al. 2016;

Havulinna et al. 2016; de Carvalho et al. 2018; Peterson et al. 2018; Nwabuo et al. 2019; Li et al. 2020a). Moreover, specific ceramide ratios and risk scores have been proposed in the literature and can predict CVD prognosis and all-cause mortality (Öörni et al. 2020).

Apart from metabolic diseases, ceramides are considered useful biomarkers and therapeutic targets for a great variety of health disorders, including multiple sclerosis, ovarian and colorectal cancer, and Alzheimer's disease (Kurz et al. 2019). They are also being studied in multiple gestational disorders, including gestational diabetes mellitus, preeclampsia, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, and intrahepatic cholestasis, all promising results. Moreover, a correlation has been reported between low ceramide concentrations in the amniotic fluid of women carrying babies with Down's syndrome (Charkiewicz et al. 2015).

Diet-Induced Responses to Ceramide Concentrations

Diet appears to be an important effector of ceramide concentration in humans and nonhuman primates, with most of the ceramide synthesis being diet-centric (Mah et al. 2021). Animals fed a western, high-fat diet demonstrate increased concentrations of ceramide (Brozinick et al. 2013) and exacerbated remodeling of cardiac ceramide C16 and C18 species (Butler et al. 2017).

Research on distinct dietary patterns and ceramide response on humans has evaluated interventions with the Mediterranean diet (MD) and the lacto-ovo-vegetarian diet (VD). Samples from the Framingham cohort revealed that greater adherence to the MD is associated with reduced C16:0 and C20:0 concentrations (Walker et al. 2019). In a randomized controlled trial (RCT) design, the PREvención con DIeta MEDiterránea (PREDIMED) followed adults with an increased CVD risk for 7.4 years, after allocating them to two active dietary interventions (MD supplemented with nuts or extra-virgin olive oil [EVOO]) or their habitual diet. Participants exhibiting a higher ceramide score assigned to one of the active intervention arms had a similar CVD risk to those with a lower ceramide score (Wang et al. 2017). On the other hand, participants with a higher ceramide score assigned to the control group demonstrated a significantly higher CVD risk. Between active and comparator arms, the recorded changes in ceramide content were not significant during the first year of intervention, indicating that the therapeutic effects of diet do not appear to be acute, requiring a great adherence duration. Researchers moved further by summing the concentration of four ceramides into one score, which was associated with a 2.18-fold higher risk of CVD in the higher quartiles (Wang et al. 2017). VD was compared against the standard medical nutrition therapy among patients with coronary artery disease (CAD) (Djekic et al. 2020): adherence to a VD induced favorable changes in the concentrations of the lipotoxic intermediates, including increased ceramide content (d18:1/16:0).

Concerning the dietary fat intake, overconsumption of saturated fat induces increases in the ceramide levels in the circulation and the liver while promoting NAFLD (Luukkonen et al. 2018; Rosqvist et al. 2019). In a comparative

effectiveness RCT (Tuccinardi et al. 2021), healthy subjects of normal weight supplemented their isocaloric diet with chocolate spread snacks, providing an additional 570 kcal/day. The chocolate spreads were identical, except for the type of fat, EVOO, rich in monounsaturated fatty acids (MUFAs) in the active intervention, and palm oil, rich in SFAs in the comparator arm. After 2 weeks of intervention, the EVOO-enriched spread induced improvements in the circulating sphingolipids profile, with reduced plasma Cer C16:0, Cer C16:0/Cer C22:0-Cer C24:0 ratio and sphingomyelin C18:0 (Tuccinardi et al. 2021).

Palmitic acid (PA) and oleic acid (OA) are considered as the yin and yang of fatty acids concerning diabetes mellitus (DM) (Palomer et al. 2018). Substitution of SFAs by OA has improved insulin sensitivity while preventing PA-driven inflammation and IR (Palomer et al. 2018). When diets enriched in PA were compared to diets rich in OA but low in PA, the latter were accompanied by lower circulating and intramuscular ceramide concentrations, paired with significant reductions in inflammatory and oxidative stress biomarkers (Kien et al. 2013).

Several clinical practice guidelines advocate for increased fish consumption to reduce CVD risk. One trial evaluated fish intake among patients with a history of myocardial infarction or unstable ischemic attack (Lankinen et al. 2009). Participants were allocated to consume four meals of fatty fish, four of lean fish, or only one fish meal per week. After 8 weeks of intervention, participants in the fatty fish arm demonstrated reduced ceramide content (Lankinen et al. 2009).

Several studies indicate the decisive role of diet and nutritional status of pregnant women at the offspring's lipidomic profile. When 4-year-old children were assessed, similar quantitative differences in the plasma concentrations were observed in those born to pregnant women with overweight and those born to mothers who were obese during gestation. Moreover, supplementation of the maternal diet with n-3 fatty acids during gestation induced a reduction in ceramide abundance, indicating that plasma ceramides are promising biomarkers of the offspring's metabolic condition (León-Aguilar et al. 2019).

In the only maternal-offspring pair RCT, maternal supplementation with n-3 long-chain polyunsaturated fatty acids during pregnancy induced notable changes in the child metabolome, including a decrease in ceramides and sphingolipids containing 18:0 and 22:0 fatty acids (Rago et al. 2019).

Obesity is associated with an increased carbohydrate intake, especially among younger populations. In a trial substituting sugar-sweetened beverages with low-fat milk in adolescent boys for a total of 3 weeks (Chiu et al. 2020), apart from the improvements in physiological outcomes, milk consumption additionally decreased plasma glucosylceramides (d18:1/C16:0) and lactosylceramides (d18:1/C16:0 and d18:1/C18:0). Thus, even a short-term intervention appears to be effective concerning the cardiometabolic risk.

Only a handful of studies have assessed supplementation with oral nutrient supplements, including complementary medicine compounds and the response regarding ceramide synthesis. Moreover, due to the recent research interest in ceramides, most results derive from post hoc analyses of RCTs. In a recent RCT, 4 weeks of red ginseng supplementation improved the ceramide profile of

postmenopausal women with hypercholesterolemia (Kwon et al. 2021). Two placebo-controlled RCTs evaluated changes in ceramide concentrations following supplementation with vitamin D. In the first (Koch et al. 2017), the retrospective analysis of the data revealed that daily supplementation with 1904 IU of vitamin D (via Vigantol oil) for 6 months improved plasma C18 dihydroceramide (dhCer; N-stearoyl-sphinganine (d18:0/18:0)) and C18ceramide (Cer; N-stearoyl-sphingosine (d18:1/18:0)) in patients with DM. Similarly, in the second trial, the post hoc analysis indicated that supplementation with vitamin D_3 for 6 weeks increased serum levels of dhCer and Cer in a dose-response manner among African Americans with overweight/obesity (Chen et al. 2020).

Apart from dietary manipulations, muscle ceramide concentrations can be independently reduced via adherence to a hypocaloric diet, bariatric surgery, or exercise (Dubé et al. 2011; Coen et al. 2015). On the other hand, any positive change in the energy balance like increasing energy intake or reducing physical activity levels, even short term, can induce a modest increase in serum and intramuscular ceramide levels, independent of IR (Reidy et al. 2018). Thus, ceramide synthesis acts as an innate switch, buffering fatty acid excess, indicative of metabolic inflexibility and dysregulated homeostasis, while aiming to protect our "thrifty genes" from overnutrition.

Conclusions

Ceramides are evolutionary metabolic signals of fatty acid overload (Summers 2020). Contemporary research suggests that determining the exact mechanisms of dysregulating ceramide metabolism may help unravel novel treatments for metabolic diseases (Öörni et al. 2020). Moreover, although ceramide reduction therapy can be targeted to treat metabolic diseases and CVD, the same appears to be the case for diet.

Difficulties in quantifying the different ceramide species in biological samples and the relatively low concentrations in both tissue and plasma (Kirwan 2013) hinder research possibilities aiming to increase our knowledge on their exact physiological role and any diet-induced responses. It is important to specify if the ceramide levels in different tissues are an independent risk factor for CVD and metabolic syndrome. Further research is required to specify the metabolic paths that can be modified with diet or pharmaceutical agents to prevent future disease onset. In parallel, a high research interest exists regarding ceramide concentrations following different dietary patterns.

Applications to Prognosis

Elevated ceramide concentrations are associated with adiposity and the development of IR, T2DM, and increased CVD risk. Adherence to a hypocaloric diet, Mediterranean diet, sugar restriction, substitution of unhealthy dietary fats, and supplementation with n-3 fatty acids and vitamin D reduce ceramide concentrations, improving prognosis.

Applications to Other Diseases or Conditions

Apart from metabolic diseases, ceramides are also considered useful biomarkers and therapeutic targets for a great variety of health disorders, including multiple sclerosis, ovarian and colorectal cancer, and Alzheimer's disease (Kurz et al. 2019). They have been studied in many gestational disorders, including gestational diabetes mellitus, preeclampsia, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, and intrahepatic cholestasis with promising results. Moreover, a correlation has been reported between low main ceramide concentrations in the amniotic fluid of women carrying babies with Down's syndrome (Charkiewicz et al. 2015).

Mini-dictionary of Terms

- ***Sphingolipids***: *A lipid class with a backbone of sphingoid bases and a set of aliphatic amino alcohols that includes sphingosine.*
- ***Thrifty genes***: *A hypothesis explaining the high prevalence of obesity and diabetes, in modern times, due to affluence.*
- ***Metabolic inflexibility***: *Inability to respond or adapt adequately to the metabolic demand.*
- ***Homeostasis***: *Maintenance of energy balance.*
- ***Ceramide synthetase***: *Enzyme participating in ceramide formation.*

Key Facts of Ceramides

- Ceramides are the simplest sphingolipid form situated at the center of sphingolipid metabolism.
- Evolutionary mechanisms equipped human cells with ceramides to buffer excess free fatty acids and ensure that intracellular concentrations remain optimal.
- In prolonged free fatty acid overload, de novo ceramide synthesis is initiated, propelling insulin resistance, hepatic steatosis, and the development of cardiometabolic diseases.
- Inhibiting ceramide synthesis is associated with adipose tissue browning and increased thermogenesis, forming a possible therapeutic pathway for metabolic disease.
- Dietary manipulations can alter ceramide concentrations, providing another means of reducing lipotoxic intermediate concentrations.

Summary Points

- *Elevated ceramide concentrations are associated with an excess in energy intake and adiposity.*

- *Dietary manipulations can be used to reduce ceramide synthesis and circulating concentrations.*
- *Inhibiting ceramide synthesis is a promising target treatment for the primary prevention of metabolic and cardiovascular diseases.*

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Part II

Micronutrients and Minerals

Urinary Arsenic as a Biomarker: Speciation Analysis for the Assessment of Dietary Exposure

9

Speciation Analysis and Implications for Dietary Exposure

Jun Yoshinaga

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Abstract

Chronic adverse health effects due to groundwater contamination of inorganic arsenic (iAs) has long been known in many parts of the world. Carcinogenic effect of iAs due to low-level dietary exposure has become a serious matter of concern even in noncontaminated regions of the world. For the effective health risk management of dietary iAs intake, a quantitative dose-response relationship has to be established based on reliable analytical epidemiologic studies. Urinary levels of iAs and its metabolites, monomethylarsonic acid (MMA) and

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dimethylarsinic acid (DMA), are a suitable biomarker of exposure, and urine analysis serves as a reliable tool of exposure assessment of individuals in such epidemiologic studies. This review summarizes a method of urine As speciation analysis, and advantages and limitations of the use of urine As species as a biomarker of exposure to dietary iAs in environmental epidemiologic studies are discussed. Use of urine As speciation as a biomarker of susceptibility is also referred.

Keywords

Arsenic · Monomethlarsonic acid · Dimethylarsinic acid · Urine · Exposure assessment · Biomarker of exposure · Biomarker of susceptibility · Cancer · Epidemiology · Risk assessment · Risk management

Abbreviations

AAS	Atomic absorption spectrometry
AB	Arsenobetaine
AC	Arsenocholine
AFS	Atomic fluorescence spectrometry
As	Arsenic
CRM	Certified reference material
DMA	Dimethlarsinic acid
DMA^{III}	Dimethylarsinious acid
DR	Diet recall
FFQ	Food frequency questionnaire
iAs	Inorganic arsenic
ICC	Intra class correlation
ICP-AES	Inductively coupled plasma- atomic emission spectrometry
ICP-MS	Inductively coupled plasma- mass spectrometry
LC	Liquid chromatography
MMA	Monomethylarsonic acid
MMA^{III}	Monomethylarsenious acid
NMSC	Non- melanoma skin cancer
SCC	Squamous cell carcinoma
TMA	Trimethylarsine
TMAO	Trimethylarsine oxide

Introduction

Arsenic (As) is a toxic metalloid and known as a poison from ancient time. Acute high-level exposure to As results in neurological and gastrointestinal symptoms which can be fatal (ATSDR 2007). Chronic exposure to excessive As has been known to be a cause of skin lesions and cancers of the lung, bladder, kidney, liver, and prostate in addition to cancer of skin (IARC 2012). Endemic occurrence of these

symptoms has been well documented among the general populations in Taiwan, China, South and Southeast Asian countries, and South American countries due to groundwater contamination of As. The number of affected people is estimated to be nearly hundred million or more in the world. The As in the groundwater in these regions of the world was mostly of natural origin, though anthropogenic origins, such as mining activity, were present in some cases in the past. Thus, health problems with As in the world are more as natural disaster than as an anthropogenic, industrial pollution at present.

The abovementioned toxicities of As are exclusively from inorganic As (iAs), i.e., arsenate (As(V)) and arsenite (As(III)). It is well known that a variety of organic forms of As are present in the environment particularly in marine biota. More than 100 organic As compounds have ever been identified in the nature. In contrast to the toxic iAs, most of the organic As compounds are much less toxic. Humans are exposed to organic As compounds mainly via the consumption of marine products, such as seafood and seaweeds. Some foods of terrestrial origin contain detectable levels of iAs and some methylated As compounds of simpler structure, e.g., dimethylarsinic acid.

For the assessment of health effects of As in humans at environmental levels, speciation analysis of As is recognized essential at present because of the great variation in toxicity of different chemical forms. Total As analysis can only be applicable to a limited situation where the sole involvement of iAs as a contaminant but not of others is clearly known.

This review summarizes analytical method of As speciation, liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS), and its application to the field of environmental health. In the field of environmental health, WHO's definition of biomarker (IPCS 1993) has been widely used: biomarker is defined as any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical, or biological. Three classes of biomarkers were identified as (i) biomarker of exposure, (ii) biomarker of effect, and (iii) biomarker of susceptibility (IPCS 1993). In this review, described is urinary As speciation analyzed by LC-ICP-MS as a biomarker of exposure to iAs, which will be more valuable in future epidemiologic researches of As exposure and cancer and/or neurodevelopment. Urinary As speciation as a biomarker of susceptibility to iAs toxicities will also be referred.

Arsenic Species in the Environment and Biota

There are many organic As compounds in the environment, mainly in marine organisms, which include methylated As (monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethyl As compounds, etc.; Fig. 1) and more complicated organic As compounds, e.g., arsenosugars and arsenolipids (Fig. 2). These organic As compounds are generally much less toxic, except for a couple of possible exceptions referred to later, when compared with the toxic iAs. The comparison of lethal dose 50 (LD_{50}) values of inorganic and some organic As

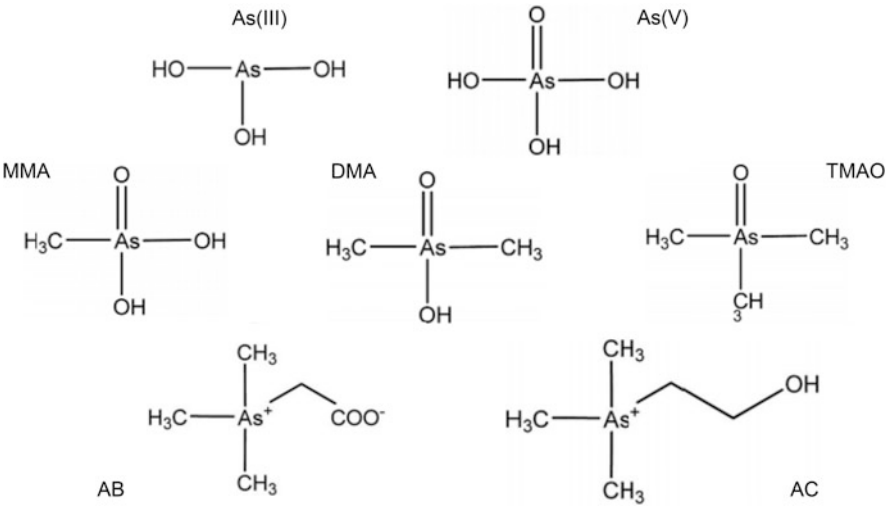

Fig. 1 Structure of arsenic species (1)

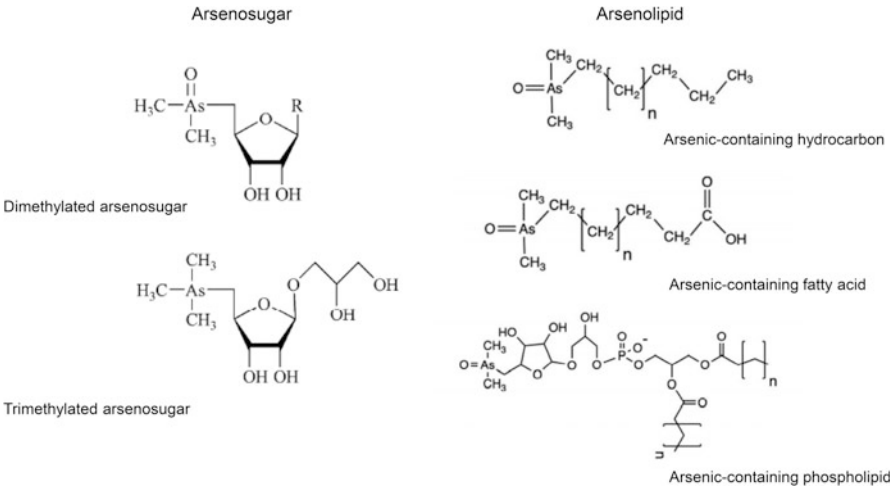

Fig. 2 Structure of arsenic species (2). R in dimethylated arsenosugar is either one of the following: H, OH, glycerol, phosphate, sulfonate, or sulfate

compounds in rodents (Kaise et al. 1985, 1989, 1992) is shown in Fig. 3. It is particularly interesting to note that LD_{50} of arsenobetaine (AB), a trimethylated As compounds commonly and abundantly found in marine animals, is as much as >10 g/kg (Kaise et al. 1985), suggesting that it is virtually nontoxic.

Arsenosugars are found in plankton and seaweed, and arsenolipids are in fish and seaweed. Some of the arsenolipid compounds found in fish and seaweed are known to have potent cell toxicity (Meyer et al. 2014), but most others do not. Occurrence of

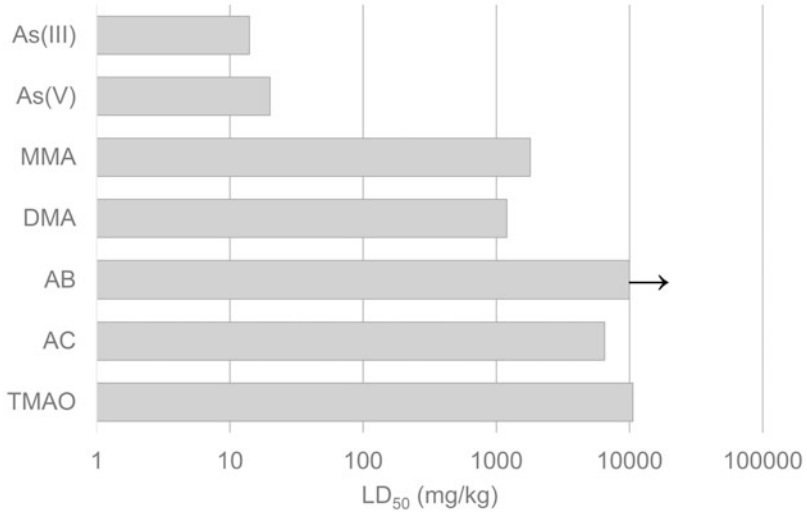

Fig. 3 Acute toxicity of arsenic species. Lethal dose 50 (LD_{50}, mg/kg) in rodents. As(III), arsenite; As(V), arsenate; MMA, monomethylarsonic acid; DMA, dimethylarsinic acid; AB, arsenobetaine; AC, arsenocholine; TMAO, trimethylarsine oxide. (Source: Kaise et al. 1985, 1989, 1992)

Table 1 Major As species in food category

Food category		Major As species
Terrestrial plant	Rice	iAs, DMA
	Mushroom	iAs, DMA, AB
	Others	iAs
Aquatic plant	Seaweeds	iAs, MMA, DMA, Assugars, As-lipids
Terrestrial animal	Meat	iAs, MMA, DMA
Marine animal	Fish	MMA, DMA, AB, AC, TMAO, As-lipids
	Shellfish	MMA, DMA, AB, TMAO, As-sugars, As-lipids
	Crustaceans	MMA, DMA, AB, AC, TMAO, As-lipids
Freshwater animal	Fish	DMA, AB

MMA monomethylarsonic acid, *DMA* dimethylarsinic acid, *iAs* inorganic As, *AB* arsenobetaine, *AC* arsenocholine, *TMAO* trimethylarsine oxide

organic As compounds other than MMA and DMA is not common in terrestrial environment and biota, where iAs is usually found at lower concentration.

Humans are exposed to various As compounds via the diet in addition to iAs from drinking water. Table 1 shows major As species in some food categories. People are exposed to these As species depending on the food choices.

Other exposure routes including inhalation and dermal exposure do not significantly contribute to aggregated As exposure of general population. Oral ingestion of soil and house dust, which were known to contain only iAs, was not a significant contributor to As exposure (Oguri et al. 2013).

HPLC-ICP-MS for Speciation Analysis of As

Speciation of As was analyzed in early days by selective extraction of iAs in hydrochloric acid into solvent followed by atomic absorption (AAS) detection (Yasui et al. 1978). Later, hydride generation (HG) technique was widely employed for speciation of inorganic and methylated As species (Welna et al. 2020). This technique utilizes different boiling points of liquid nitrogen-trapped hydrides of different As compounds or gas chromatographic separation of hydrides of the As compounds. Atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS) were typically used as a detector of As in HG technique. However, this HG technique was applicable only to iAs, MMA, and DMA speciation because these are the only compounds that form volatile hydride by sodium borohydrate reduction. Other organic As compounds of biological or environmental significance, e.g., AB, do not form hydride: speciation analysis based on HG technique had only a limited value in As speciation of biological and clinical samples unless chromatographic separation and As species digestion are preceded (mentioned later in this section).

Subsequently, liquid chromatographic separation with inductively coupled plasma-atomic emission spectrometry (ICP-AES) detection was developed for As speciation in the early 1980s (Morita et al. 1981). They employed cation and anion exchange columns for the separation of 5 As species including AB and applied anion exchange to hijiki extract. Use of LC is much more advantageous over HG technique because As compounds that do not form hydride could be speciated without tedious sample pretreatment. The ICP-AES detection was quickly replaced by ICP-MS detection because the latter was much more sensitive for the detection of As than was ICP-AES. Detection limit of As by LC-ICP-MS was around 0.1 μg/L, which was lower by 100 times than that by ICP-AES. The improvement of detection limit made LC-based technique applicable to urinary As speciation analysis of non-exposed individuals.

However, use of ICP-MS as a detector of LC had a drawback: it limited the composition of mobile phase of LC because ICP-MS did not favor the introduction of high-concentration salts or organic solvent. This is because introduction of salts or organic solvent makes ionizing source (argon plasma) unstable and buildup of salt or carbon (soot) at the interface between ICP and mass spectrometer, which significantly deteriorates stability and sensitivity of measurement. For this reason, ion exchange or reversed phase column with mobile phase containing dilute salts has been favored for As speciation with ICP-MS detection.

Anion or cation exchange separation has widely been used for separation of iAs, MMA, and DMA for ICP-MS detection (Ardini et al. 2020; Reid et al. 2020). Diluted salts are used for mobile phase (e.g., 10 mM ammonium phosphate). Cation exchange separation was used for the speciation of trimethylated and tetramethylated As compounds. Ion-pair, reversed phase separation has also been the method of choice for the separation and determination of iAs, methylated compounds, and arsenosugars. Employment of three different separation modes including two ion-pair, reversed phase separations coupled with ICP-MS detection enabled

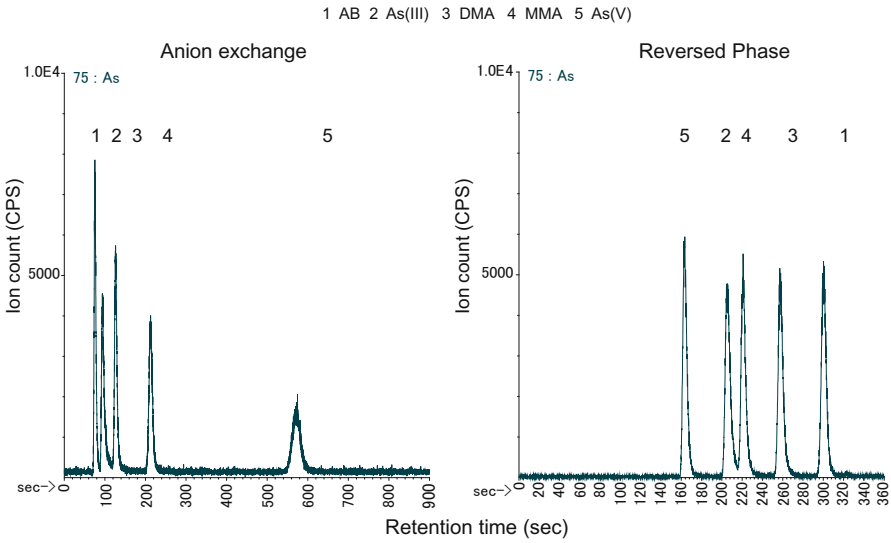

Fig. 4 Chromatograms of arsenic species standard by anion exchange and reversed phase separation modes. Anion exchange: Hamilton PRP X-100 (10 μm, id 4.1 × 150 mm), 10 mM $(NH_4)_2HPO_4$ (pH 8.25). Reversed phase: Shiseido CAPCELL PAK C_{18} (3 μm, id 4.6 × 150 mm), 10 mM Sodium 1-butanesulfonate/4 mM malonic acid/4 mM tetramethylammonium hydroxide/0.05% methanol (pH 3.0) (Narukawa and Chiba 2010). Standard mixture solution (20 μL injection) containing 20 μg As/L each of (1) AB, (2) As(III), (3) DMA, (4) MMA, and (5) As(V). For abbreviations, see legend to Fig. 3. (Chromatograms provided by Dr. T. Narukawa, National Institute of Advanced Industrial Science and Technology, Japan)

full separation of 15 As compounds, and it was applied to As speciation of human urine (Shibata and Morita 1989). Figure 4 shows comparison of anion exchange and reversed phase separations of five major As compounds. Column used for anion exchange was PRP X-100, the most widely used one, and that for reversed phase was C_{18} ODS with mobile phase composition originally developed by Shibata and Morita (1989). Since the elution order is As(III), AB, MMA, DMA, As(V) in anion exchange (Fig. 4 left), peak width of As(V) becomes wider, leading to lower signal to noise. In addition, As(III) is not fully separated from other cationic compounds (e.g., trimethylarsine oxide and tetramethylarsine), and co-elution can occur, requiring combined use of cation exchange for the full separation of wider range of compounds. For the latter, effort has been made to make separation better by careful selection of composition and pH of mobile phase. On the other hand, ion-pair, reversed phase separation provides full separation of toxicologically important species from cationic As compounds. Thus, generally speaking, reversed phase separation is better than anion exchange in terms of accurate determination of As(III) and As(V), important analytes in terms of health effect assessment.

HG-based speciation technique was developed by including online oxidation module in the system to render non-hydride-forming As compounds to hydride-forming compound. Liquid chromatographically separated As species were

individually oxidized to form hydride-generating compound (e.g., iAs) online, which were subsequently detected by HG-based analytical method. Thus, complicated system, e.g., LC-online oxidation-HG-ICP-MS (or other detectors), was developed (e.g., Nakazato and Tao 2006). Chemical reagent, microwave, or ultraviolet radiation, sometimes a combination of these, was used for online oxidizer in such system. The most remarkable merit of this complicated system was higher sensitivity, e.g., 1–2 ng/L of detection limit, which resulted from higher sample introduction efficacy of gaseous hydride to the detector as well as low background due to freedom from coexisting components.

Urinary As Species as a Biomarker of Exposure

Since toxicity and carcinogenicity are expected virtually for iAs exposure only for the moment, human exposure assessment is necessary for iAs but not for other organic As species. It is well known that absorbed iAs is metabolized to MMA and DMA in the liver in humans by the combination of oxidation, reduction, and methylation (Watanabe and Himeno 2013; Stýblo et al. 2021). Although methylation was previously regarded as detoxifying process of toxic iAs in organisms, it turned out it's not necessarily the case because intermediate metabolites produced during methylation process, monomethylarsenious acid (MMA^{III}) and dimethylarsinious acid (DMA^{III}), were found to be more toxic than iAs. The MMA^{III} and DMA^{III} in cells are eventually oxidized to less toxic MMA and DMA (MMA and DMA without superscript denote MMA^{V} and DMA^{V}, respectively, throughout this review) in the organisms and are excreted.

Urine As concentration has been used for biomarker of iAs exposure in humans because absorbed iAs is excreted in urine as iAs, MMA, and DMA with a relatively short biological half-time (1–2 days, Buchet et al. 1981). Urinary total As concentration was used in earlier studies, and it is still acceptable as a biomarker of exposure when source of exposure is known and species exposed is limited to iAs. For instance, for a population living in groundwater-contaminated area and who do not consume marine products, the exposed As species is almost exclusively inorganic except for a minor portion of DMA from crops (Table 1). In this case, As in urine is expected to be composed of iAs and its metabolites MMA and DMA: thus total As concentration is equal to the sum of As species resulting from iAs exposure, and total As analysis is justified in this case. However, for a population who consume marine products, such as seafood and seaweed, they are exposed to a variety of nontoxic As species; therefore, urinary total As analysis is never an option for As exposure assessment because organic As species are also excreted in urine unchanged or after metabolism. Speciation analysis of As in urine, namely, iAs, MMA, and DMA, is essential in such populations for the assessment of toxic iAs exposure. Here, iAs means the sum of As(III) and As(V) because it is difficult to completely control analytical artifact that induces valency conversion, e.g., oxidation of As(III) to As(V) or reduction of As(V) to As(III), during storage and/or analysis of urine sample (Crecelius and Yager 1997). In this review only the sum of As(III) and As(V) is dealt with and expressed as "iAs."

As a reliable biomarker of exposure to iAs, urinary concentrations of iAs, MMA, and DMA should be measured and summed for the quantitative estimation of iAs exposure level. Although MMA and DMA are contained in some foods of terrestrial origin, the levels are generally so low that the direct contribution from dietary MMA and DMA to urinary MMA and DMA has been ignored, and use of iAs + MMA + DMA in urine has been justified as a biomarker of iAs exposure. However, for a population consuming marine products, urinary iAs + MMA + DMA cannot be used as a biomarker of iAs exposure because appreciable contribution is expected from DMA produced from metabolism of some organic As compounds. Arsenosugars are the typical example: DMA was the most abundant metabolite excreted from arsenosugar-dosed humans (Ma and Le 1998; Francesconi et al. 2002). This meant that urine iAs + MMA + DMA could overestimate iAs exposure in seaweed-consuming populations due to DMA metabolized from arsenosugars contained abundantly in seaweeds. DMA is also produced from the metabolism of arsenolipids (Schmeisser et al. 2006), which are known to be contained in seaweeds and seafood.

Hata et al. (2016) suggested the use of iAs + MMA as a biomarker of iAs exposure in such population. They found a highly significant positive correlation ($r = 0.962$, $p < 0.0001$) between iAs + MMA and iAs + MMA + DMA in urine samples from Bangladeshi people ($n = 330$) who do not consume marine products. The authors successfully applied urinary iAs + MMA for the assessment of occupational inhalation exposure to iAs in Japanese workers, who habitually consume substantial amount of seafood and seaweeds. Thus, urinary iAs + MMA + DMA or iAs + MMA is regarded as a biomarker of iAs exposure depending on whether the target population consume marine products or not.

Due to the widespread notion that speciation information is essential, recent human biomonitoring programs include not only urinary total As but also As species. Table 2 shows urinary As speciation results of the US, French, and Canadian

Table 2 Average levels of arsenic species reported in human biomonitoring programs (μg/L otherwise indicated)

Country	N	Year of sampling	As(III)	As(V)	MMA	DMA	AB	iAs + MMA +DMA	Others	Ref
USA	2568	2003–2004	<1.0	<1.2	<0.9	3.90	1.00		AC <0.6 TMAO <1.0	Caldwell et al. (2009)
USA	9537	2003–2014			0.73	3.84	1.98			Wang et al. (2018)
France[a]	1515	2006–2007						3.53		Saoudi et al. (2012)
Canada	2615	2016–2017	0.36	<0.14	0.40	3.1		4.1	AB +AC 1.3	Health Canada (2019)

For abbreviations, see footnote to Table 1

[a]Unit: μg/g creatinine

Table 3 Analytical results of arsenic species in certified reference materials of urine matrix. (unit: ng/mL)[a]

	NIST SRM 2669 Frozen Human Urine Level I		NIST SRM 2669 Frozen Human Urine Level II	
	Certified	Measured ($n = 8$)	Certified	Measured ($n = 8$)
As(V)	2.41 ± 0.30	3.81 ± 0.13[b]	6.16 ± 0.95	11.43 ± 0.24[b]
As(III)	1.47 ± 0.10		5.03 ± 0.31	
MMA[c]	1.87 ± 0.39	1.83 ± 0.08	7.18 ± 0.56	6.97 ± 0.35
DMA[c]	3.47 ± 0.41	3.52 ± 0.19	25.3 ± 0.70	25.3 ± 0.30
AsB[c]	12.4 ± 1.9	12.5 ± 0.55	1.43 ± 0.08	1.46 ± 0.02

[a]Yoshinaga J and Narukawa T (2020). (Reproduced with permission)
[b]Sum of As(III) and As(V) on the chromatogram was shown because transformation of valence could be possible
[c]Abbreviation: *MMA* methylarsonic acid, *DMA* dimethylarsinic acid, *AsB* Arsenobetaine

populations (Caldwell et al. 2009; Saoudi et al. 2012; Wang et al. 2018; Health Canada 2019). Anion exchange LC-ICP-MS was used for the determination in these programs. As can be seen from this table, As(III), As(V), and MMA were hardly detectable in the US, French, and Canadian populations: they were detectable only <10% of the subjects of each population. DMA and AB were detectable. This was due to lower abundance of iAs and MMA in urine, suggesting that the populations were, on average, not exposed to excessive iAs. Moderate sensitivity of LC-ICP-MS system might also be the reason of low detectability. Detection of AB in these programs indicated that the US, French, and Canadian populations consume some seafood: this implies that a fraction of the DMA detected in urine might be from the metabolism of arsenolipids in seafood and thus overestimation of iAs exposure levels could occur when iAs + MMA + DMA was used as a biomarker of exposure.

Analytical quality assurance of LC-ICP-MS analysis is a relevant issue when using the urinary concentrations of iAs, MMA, and DMA as a biomarker of iAs exposure. Certified reference materials (CRMs) from the National Institute of Standards and Technology (NIST), USA, are widely used for this purpose. Table 3 shows an example of analytical quality assurance of urinary LC-ICP-MS analysis for iAs exposure assessment based on NIST CRMs (Yoshinaga and Narukawa 2020). Urine-based CRM for As speciation is also available from the National Institute for Environmental Studies, Japan (NIES CRM No. 18 Human Urine). Presentation of the analytical results of urine-based CRM is required for any studies that involve urinary As speciation analysis.

Urinary iAs Speciation Analysis in Environmental Epidemiology

Intake level of iAs is crucial information for the assessment of health risks of individual and population. In earlier epidemiologic studies on the health effects of groundwater As contamination, total As levels in drinking water were used as an exposure proxy of a population. Concentration of total As in public well water was used as exposure proxy

of all the people who use the well in some epidemiologic studies. Apparently, this exposure assessment did not provide accurate exposure of individuals and population because water consumption can be variable among individuals of the target population, and this did not take into consideration possible intake from diet. However, this approach has been justified because (1) this could provide cost-effective semiquantitative estimate of exposure levels of large number of individuals under the situation of urgent research needs and (2) intake of As from drinking water was assumed to be much greater than that from diet in groundwater-contaminated region. This semiquantitative approach was applicable only to the exposure assessment of ecological epidemiologic studies in groundwater-contaminated area.

Public awareness of carcinogenetic effect of iAs has stimulated concern over the cancer risk of general populations in the regions where groundwater is not contaminated. More quantitative approach of exposure assessment on individual basis has become necessary to establish a reliable dose-response relationship in an analytical epidemiologic study. Establishment of a dose-response relationship is indispensable for accurately assessing health risk. For the quantitative assessment of iAs exposure, not only iAs from drinking water but also the dietary iAs intake must be assessed in the regions where groundwater contamination is not present. There are two quantitative approaches for the assessment of dietary intake of a chemical. Food frequency questionnaire (FFQ) or diet recall (DR) coupled with an appropriate food composition table is one of the approaches. This approach has often been taken in a large-scale cohort study because it is based on questionnaire and less time-, cost-, and labor-consuming for obtaining semiquantitative exposure/intake information from a large number of subjects. There were several studies that estimated iAs intake of individuals by FFQ or diet recall approach (Signers-Pastor et al. 2008; Sawada et al. 2013; Wong et al. 2013).

A duplicate diet study is another approach, which measures the concentration of the target chemical in a duplicate diet sample collected from subject to calculate daily intake on individual basis. It provides fully quantitative intake information of the day of diet sampling; however, apparently it is much more time-, cost-, and labor-consuming than FFQ/DR. The most problematic point is that duplicate diet approach requires extensive sample pretreatment including sample collection, homogenization, extraction of iAs, as well as subsequent measurement. Thus, duplicate diet approach is not practical for assessing exposure in a large-scale epidemiologic study. Daily iAs intake estimation of a population based on duplicate diet approach has been reported in several publications (Ruangwises et al. 2011; Hayashi et al. 2019).

Use of urinary levels as a biomarker of iAs exposure has advantages over the estimation based on diet iAs. Urine analysis is much less time- and labor-consuming than duplicate diet because it does not require extensive sample pretreatment but only dilution. It is possible to measure a large number of samples. Moreover, urine concentration of iAs + MMA + DMA reflects internal dose of iAs, MMA, and DMA and includes contributions from both diet and drinking water. It is not affected by, e.g., erroneous recall in FFQ/DR approach or missing food items in duplicate diet approach. Increasing number of epidemiologic studies has been published in which levels of urinary iAs species were used as an exposure metrics. Gilbert-Diamond

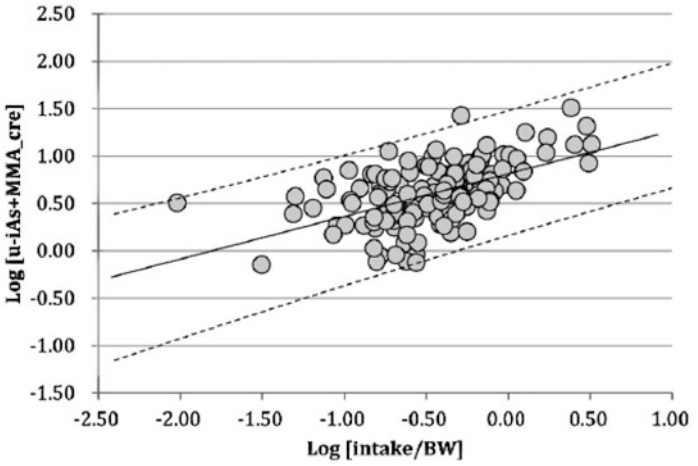

Fig. 5 Scatter plot of inorganic arsenic intake and sum of urinary levels of inorganic As and monomethylarsonic acid. (Reproduced from Yoshinaga and Narukawa (2020) with permission)

et al. (2013) conducted a case-control study on incidence of squamous cell carcinoma (SCC) and urinary iAs, MMA, and DMA levels in New Hampshire, USA. Three hundred and twenty-three case and 319 control subjects were involved in the statistical analysis. The mean concentration of urinary iAs + MMA + DMA of case and control was 5.27 and 4.76 μg/L, respectively, indicating that both cases and controls were not excessively exposed (see Table 2). The authors found a positive association between SCC and urinary iAs + MMA + DMA concentration, as well as that between SCC and MMA, and SCC and DMA. These results indicated that iAs exposure could be involved in carcinogenesis of skin at the levels general public are exposed (Fig. 5).

Kim et al. (2017) also conducted a case-control study on the association between As exposure and non-melanoma skin cancer in Korea. Geometric mean urinary iAs + MMA + DMA concentration of the patients was 34.52 μg/L ($n = 124$) and higher than the USA mean of Gilbert-Diamond et al.; this must be due to higher DMA (32.78 μg/L) in these Korean study subjects. Urinary iAs concentrations were significantly higher in the cases (0.140 μg/L) than in controls (0.055 μg/L), but the difference was not significant for organic As (MMA + DMA). A significant difference was found when basal cell carcinoma and SCC cases were separately tested. Higher DMA concentrations of the cases and controls in this study suggested the contribution of seafood and/or seaweed consumption, as Koreans are known to consume marine products. If this was the case, use of iAs + MMA + DMA was not appropriate as a biomarker of iAs exposure in this study, and this might be in part the reason of the absence of significant difference in urinary iAs + MMA + DMA between cases and controls.

Signers-Pastor et al. (2019) cross-sectionally examined the relationship between iAs exposure and neuropsychological development among children in Spain. Mean

urinary concentration of iAs + MMA + DMA was 4.85 μg/L ($n = 361$) indicating that the children had low-level exposure. The authors found a negative association between urinary iAs + MMA + DMA and some of the motor functions of children. Since urinary AB concentration was rather high for the children (mean, 67% of iAs + MMA + DMA + AB), some of the DMA must have been metabolic product of arsenolipids so that use of iAs + MMA + DMA as a biomarker of exposure to iAs might be erroneous.

Quantitative Relationship Between Urinary As Speciation and Dietary Intake

Since there is no animal model for some of the adverse effects of iAs, e.g., cancer and neurobehavioral development, a threshold value, a value below which unacceptable adverse effect is not expected, has to be obtained from dose-response relationship established in epidemiologic studies. When urinary As concentrations are used as an exposure metrics in the epidemiologic studies, then the threshold value would be obtained as a urinary concentration. Risk managers have to express the threshold as daily intake, such as tolerable daily intake or reference dose, for the protection of public health. Therefore, it would become necessary to convert urinary iAs concentration to daily intake of iAs. Quantitative relationship between iAs intake and urinary iAs and metabolites (MMA and DMA; iAs, MMA, and DMA are collectively designated "iAs species" hereafter) concentrations has not been extensively investigated to date.

Uchino et al. (2006) estimated total As intake from drinking water and diet and urinary total As concentrations in 147 subjects living in West Bengal, India, where groundwater As contamination is known. Sum of total As intakes from drinking water and diet was estimated to be 37.1–1098 μg/person/day. Although total As was measured, it was estimated to be mostly iAs. They found a positive correlation between urinary As and estimated daily As intake ($r^2 = 0.134$, $p < 0.001$). They presented a regression equation: [daily As intake] = 0.341 × [urinary As conc] + 244.106. It is not known if transformation of urinary As concentration or daily As intake was in fact needed or not in calculating Pearson correlation coefficient and regression equation. This regression equation may be used for converting urinary As (most probably iAs + MMA + DMA) to dietary iAs intake in exposed population.

Yoshinaga and Narukawa (2020) measured iAs intake and urinary As species concentrations of 150 Japanese subjects. The general Japanese is known to be exposed to iAs via the consumption of rice and hijiki seaweed (Oguri et al. 2014), while drinking water does not significantly contribute to iAs intake. The intake of iAs was obtained by duplicated diet approach, and urinary speciation analysis was performed to obtain the concentrations of iAs species and other organic As compounds. Since the Japanese habitually consume seaweed and seafood, urinary iAs + MMA concentrations but not iAs + MMA + DMA were related to daily intake of iAs in this study. Geometric mean daily intake of iAs was 20.5 μg/person/day or

0.349 μg/kg/day, and median urinary iAs, MMA, and DMA were 2.66, 2.63, and 28.2 μg/L (specific gravity-corrected), respectively, for the 150 subjects. They found a significant positive correlation between the urinary iAs + MMA and daily iAs intake ($r = 0.544$, $p < 0.001$, Fig. 4) and obtained a regression equation: $\log_{10}$[daily intake] $= 0.451 \times \log_{10}$[iAs + MMA] + 0.814 where iAs + MMA is a creatinine-corrected concentration (μg As/g creatinine). The equation was also obtained for a specific gravity-corrected concentration of iAs + MMA as follows: $\log_{10}$[daily intake] $= 0.400 \times \log_{10}$[iAs + MMA] + 0.899.

Quantitative relationship between intake and excretion of iAs has to be investigated for a variety of situations, e.g., contaminated or non-contaminated, marine food eater or nonmarine food eater, etc., because the knowledge is population-specific and would be relevant for managing health risks in the future.

Problem in Using Urinary As Speciation as a Biomarker of Exposure

One important issue in using urinary iAs species concentration as a biomarker of exposure to iAs is that urinary concentration reflects only the levels of recent exposure to iAs because biological half-time of iAs species is not long. In contrast to this fact, what is needed is an assessment of long-term exposure to iAs of individuals when exposure is related to incidence of cancer and other chronic pathological conditions.

To what extent levels of urinary iAs species reflects long-term exposure was investigated by Kile et al. (2009). They measured iAs, MMA, and DMA concentrations in urine samples repeatedly collected from 196 subjects for 3 months in Bangladesh. The median urinary iAs, MMA, and DMA concentrations were 3.8, 2.6, and 22.8 μg/L, respectively, which were higher than corresponding average concentrations obtained in human biomonitoring as shown in Table 2. This is because the subjects in Kile et al. were exposed to groundwater As contamination. The levels of urinary iAs species were relatively constant within-subject, and intraclass correlation (ICC) coefficients were 0.35–0.49. This result indicated that iAs species concentration in a single spot urine sample collected from an individual represents long-term iAs exposure levels of the individual.

In contrast, ICC coefficient of urinary iAs + MMA concentrations collected from Japanese subjects over 4–5 months was 0.15, indicating that within-subject variation was much greater than between-subject variation (Oguri et al. 2012). The discrepancy between the results of Bangladeshi and Japanese studies was due to the difference in iAs exposure pattern between the two: In Bangladesh, subjects were exposed to drinking water iAs, while in Japan, people were exposed to dietary iAs particularly from hijiki seaweed that is usually eaten as a side dish. Hijiki seaweed is well known to contain high levels of iAs (up to 100 mg/kg dry; Yasui et al. 1978; Almela et al. 2006) and consumed almost exclusively in Japan. Frequency of hijiki seaweed consumption in Japan was reported to be 2–3 times a month. Therefore, iAs intake is expected to elevate only on the day hijiki seaweed is eaten. Exposure to drinking water iAs must be more constant than iAs intake from hijiki seaweed within-individual.

Thus, usability of urinary concentrations of iAs species as a biomarker of exposure can be different depending on the exposure pattern of the subject population. It must be confirmed if the exposure pattern can be regarded as constant when urinary iAs species are used as a biomarker of iAs exposure. Oguri et al. (2012) proposed four repetitions of urine sampling measurement for the estimation of long-term iAs intake of the Japanese based on ICC values obtained in their study.

Urinary As Speciation as a Biomarker of Susceptibility

In this review, urinary As speciation has been discussed in terms of biomarker of exposure to dietary iAs. It must be noted that urinary As speciation has also been used as a biomarker of susceptibility, a term according to WHO's definition (IPCS 1993), and more and more attention has been paid to this aspect in analytical epidemiologic studies.

Not only the concentration of iAs species but also the percentage MMA (%MMA) or DMA (%DMA) of urinary iAs + MMA + DMA was found to be associated with a variety of pathological conditions including cancers in the previous studies (Tseng 2007). Pierce et al. (2013) found a significant association between percent iAs or %MMA and skin lesion, while negative association between %DMA and skin lesion in As-exposed Bangladeshi subjects ($n = 2060$). Gamboa-Loira et al. (2017) carried out a meta-analysis, involving 13 non-ecological epidemiologic studies, on the association between urinary %MMA or %DMA and risk of cancers. They found consistent positive associations between %MMA and incidence of various cancers (e.g., bladder and negative association between %DMA and some cancer). Thus, association of higher %MMA with the elevated incidence of pathological conditions has been consistently found in many epidemiologic studies, while the association of lower %DMA with elevated disease risk was less consistent.

The %MMA and %DMA are regarded as a measure of methylation capacity of absorbed iAs: high %DMA and low %MMA represent enhanced methylation capacity, while low %DMA and high %MMA represent low methylation capacity. The abovementioned epidemiologic studies indicated that an individual with low methylation capacity is likely to develop skin lesion and cancers as a result of iAs exposure. It was speculated that low methylation capacity (=high urinary MMA) was associated with more occurrence of MMA^{III} in the cells, which is subsequently oxidized and excreted in urine as MMA (Tseng 2007). Lower methylation capacity thus results in more adverse effects. Interindividual variation in the methylation capacity in a population was attributed to genetic and environmental factors (Tseng 2009; Agusa et al. 2011). The %MMA and %DMA are derived from urinary As speciation analysis and taken as a biomarker of susceptibility to iAs toxicity/carcinogenicity.

Thus, along with iAs exposure levels, methylation capacity of individuals is now regarded as indispensable information in the epidemiologic assessment of toxicity/carcinogenicity of iAs. Speciation analysis of urinary As serves both as a biomarker of exposure and biomarker of susceptibility.

Concluding Remarks

More emphasis will be placed on conducting analytical epidemiologic studies on low-level dietary iAs exposure and cancers and other pathological conditions. Exposure information of individuals in the subject population is indispensable in such studies, particularly to establish a quantitative dose-response relationship between exposure and occurrence of adverse effects. Urinary As speciation analysis will be the unique method of choice for such studies because urinary concentration of iAs + MMA + DMA or iAs + MMA is the most appropriate biomarker of exposure to iAs. Furthermore, speciation analysis provides %MMA and %DMA, a biomarker of susceptibility, that should be included in the assessment of adverse effects. In doing so, dietary habit of the subjects must be cautiously considered for the selection of a suitable biomarker of exposure, e.g., iAs + MMA should be selected in place of iAs + MMA + DMA if the subjects consume marine food. For this consideration, measurement of AB, in addition to iAs species, is recommended as a marker of seafood consumption. It should also be considered if multiple urine sampling is required or a single spot urine sampling is sufficient for assessing long-term exposure levels. These mean that dietary habit of the subjects or a population must be carefully assessed when exposure is to be assessed.

Applications to Other Diseases or Conditions

In this chapter, reviewed is an application of urinary As speciation analysis to an analytical epidemiologic study for the establishment of dose-response relationship between inorganic As exposure and cancer, neurodevelopment, and other chronic pathological conditions. This technique has also been applied to clinical settings where internal iAs kinetics monitoring for diagnosis and treatment of acute As poisoning cases and acute promyelocytic leukemia (APL) patients dosed with arsenic trioxide for therapeutic purpose are required.

Acute arsenic poisoning cases due to accidental, suicidal, and criminal situations have been known worldwide. Arsenic speciation analysis of urine and serum has been applied to the cases for diagnosis and monitoring (e.g., Yoshimura et al. 2011). Adverse health outcomes among residents of a region in Japan due to diphenylarsinic acid (DPAA) poisoning were known. DPAA was a decomposition product of warfare agent produced during World War II in Japan, and the DPAA-containing waste was buried underground of the region for unknown reasons and the waste-contaminated groundwater of the region (Ishii et al. 2004). LC-ICP-MS analysis of biological fluids of the patients was developed and applied for the clinical investigation of DPAA-affected patients (Shibata et al. 2004).

In 1996, intravenous administration of arsenic trioxide (As_2O_3, 0.16 mg/kg dose) was found effective for the treatment of APL. Arsenic therapy is now considered as an effective therapeutic choice for APL patients resistant to all-trans retinoic acid therapy, the standard therapy of APL. Serum and/or urine As speciation analysis is a routine analytical method for monitoring of administered patients (Wang et al. 2004; Fukai et al. 2006).

Mini-Dictionary of Terms

Arsenobetaine. The most well-known organic arsenic compound. It was first purified and identified in 1977 in the muscle of Western Rock Lobster. Fishes and crustaceans contain AB at high concentration. It is nontoxic and humans excrete AB unchanged into urine quickly.

Arsenosugars. Major water-soluble organic arsenicals found in seaweeds. It was first identified in 1981. Arsenosugar contains 5-deoxypentose moiety and an arsinoyl or arsinothioyl group to C5 atom (see Fig. 2). Considered as nontoxic.

Arsenolipids. The presence of lipid-soluble forms of arsenic has long been known in marine animals and seaweeds, but identification and characterization of the compounds have recently been done. Arsenic-containing fatty acids, hydrocarbons, and phospholipids are the major arsenolipids studied so far (see Fig. 2). Most of the compounds are not toxic but some of the hydrocarbon types are found cytotoxic.

Hijiki. *Sargassum fusiforme*. It is a unique seaweed in that it contains inorganic arsenic at elevated concentration. Other seaweeds contain arsenosugars and methylated arsenicals. The Japanese traditionally consume hijiki. In 2004, UK government announced not to eat hijiki because it contains inorganic arsenic.

Methylation. Methylation of inorganic arsenic has been considered a detoxifying process of organisms because methylated arsenic is much less toxic than inorganic arsenic. Methylation was first postulated in the 1940s by reduction of arsenic atom followed by oxidative methylation (Challenger pathway). Recent evidence suggests that methylation of trivalent As atom can occur with the conjugation with glutathione. Production of highly toxic MMAIII and DMAIII during either pathway indicated that methylation is not simply a detoxifying process but it can activate arsenic toxicity at the same time.

Key Facts of Urinary Arsenic as a Biomarker: Speciation Analysis for the Assessment of Dietary Exposure

Key Facts of Urine As Species as a Biomarker of Exposure

- Humans are orally exposed to a variety of As species via drinking water and diet.
- In human urine, inorganic As (iAs), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) are detectable after iAs exposure.
- Other organic As compounds (e.g., arsenobetaine) are detectable in urine of marine food eaters.
- There is a highly significant positive association between sum of urinary iAs, MMA, and/or DMA levels and dietary iAs exposure levels.
- Significant associations were found between the levels of urinary iAs species and incidences of cancer and other pathological conditions in the previous epidemiologic studies.

- The %MMA and %DMA in urine as a methylation capacity of an individual are increasingly attracting interest in terms of its association with occurrence of pathological conditions.

Key Facts of Urinary As Speciation Analytical Method

- Liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) is a suitable method of choice for urine As speciation analysis.
- Anion exchange mode is used commonly, but reversed phase mode provides better separation of iAs species in human urine.
- Sensitivity for iAs species of LC-ICP-MS (detection limit around 0.1 μg As/L) is moderate for accurately determining iAs and MMA in urine of nonexposed subjects.
- Use of hydride generation module in LC-ICP-MS system can enhance the sensitivity by more than one order of magnitude.

Summary Points

- Inorganic arsenic (iAs) is a human carcinogen as well as the cause of other pathological conditions.
- Adverse health outcomes have been found in many parts of the world where groundwater iAs contamination is present.
- A biomarker of exposure to iAs is necessary for establishing a dose-response relationship in humans by epidemiologic studies of population who is exposed to low-level dietary iAs.
- Sum of the concentrations of urinary iAs and its metabolites, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), namely, iAs + MMA + DMA, is a suitable biomarker of exposure to iAs.
- Liquid chromatography combined with inductively couple plasma-mass spectrometric detector (LC-ICP-MS) is a suitable analytical method for arsenic speciation.
- The number of analytical epidemiologic studies involving urinary LC-ICP-MS analysis for biomarker of exposure is increasing and will be more in the future.
- Use of urinary As speciation as a biomarker of exposure has a couple of cautions, i.e., interference of arsenosugar- and/or arsenolipid-derived DMA in the urine of marine products eaters and representativeness of spot urine iAs species as a long-term exposure.
- Urinary As speciation can provide methylation capacity of an individual, which is now recognized as a relevant factor for occurrence of iAs-derived pathological conditions.

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Use of Histology in Nutrition

10

Zinc and Arsenic Studies

Roobee Garla

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Abstract

Chronic exposure to arsenic from groundwater is one of the major health concerns in several regions around the world. Binding of arsenicals with thiol moiety of protein/peptide and arsenic induced oxidative stress are the major mechanisms that are responsible for its toxicity. Due to inhibition of important

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enzymes, arsenic can alter multiple cellular processes that lead to major histopathological alterations in different tissues. Therefore, histology can be employed for the identification of the hallmark features of arsenic induced toxicity. The acceptable combating strategies for arsenic toxicity include either the reduction of arsenic concentrations at the source or to opt for the remedial methods after the exposure. Considering the economical aspect and remedial potential, the use of nutritional supplements to combat arsenic toxicity is better and rational approach especially for developing countries. Hence, Zinc (Zn), a redox-inactive essential metal, can be suitable for this purpose. Zinc helps to maintain cellular functions because of its prominent role in antioxidant network through multiple mechanisms. For that reason, the role of dietary zinc in combating the arsenic toxicity has been thoroughly studied. The histological analysis provides direct evidence for establishing the function of zinc in ameliorating the toxic effects of arsenic. This chapter provides a detailed review regarding the use of histological investigations in identifying the trademark features of arsenic induced toxicity and validating the protective effect of zinc in different organs and animal models.

Keywords

Arsenic · Toxicity · Oxidative stress · Thiol binding · Histology · Different tissues · Remedial approaches · Nutritional supplements · Antioxidants · Zinc

Abbreviations

Arsenic	As
b.w	Body weight
IARC	International Agency for Research on Cancer
kg	Kilograms
mg	Miligrams
ROS	Reactive Oxygen Species
WHO	World Health Organization
Zinc	Zn

Introduction

Over the last half century, various human activities have brought many chemical and toxic substances into the environment that are initially not the part. Arsenic (As) is one of them. As is a toxic metalloid and classified as a human carcinogen by International Agency for Research on Cancer (IARC) (Shen et al. 2013). The consumption of As contaminated water having As concentration greater than 10 $\mu g l^{-1}$, (i.e., World Health Organization (WHO) maximum permissible value) is leading to adverse effects in more than 230 million people across the globe (Shaji et al. 2021). In India alone, more than 70 million people (from 20 states and 4 UTs) are currently at the risk of As exposure through drinking water (CGWB 2018).

The toxicity of As and its compounds is dependent on their chemical composition and oxidation state (Shen et al. 2013). As(V) behave as a molecular analogue of phosphate, whereas, As (III)) have the propensity either to directly interact with the sulfhydryl groups in proteins or indirectly through the generation of reactive oxygen species (ROS) (Flora 2011). Long-term exposure of As is associated with widespread health effects ranging from disorders of skin, hypertension, neurological symptoms, cardiovascular, excretory, reproductive disorders, diabetes, and cancer (Tchounwou et al. 2019).

The plausible mitigation strategies include either the reduction of arsenic concentrations at the source or to opt for the remedial methods after the exposure. The therapeutic approaches developed includes strategy to increase the antioxidant capacity of cells or sequestration of As with the help of thiol containing chelators. Because of the financial burden of water purification and side effects of regular use of chelators, the use of nutritional supplements to combat As toxicity is better and rational approach for developing countries. Many nutritional antioxidants like vitamins, amino acids, and phytochemicals have shown promising results in ameliorating the toxic effects of As in different tissues by restoring the antioxidant defenses of the cells (Pace et al. 2017). In this context, the antioxidant properties of essential metal, Zinc and Selenium (Se), may play a protective role in case of As toxicity (Garla et al. 2021; Zwolak 2020).

Zinc (Zn) is one of the most copious essential transition metal, lacking biological redox activity (Vasak and Hasler 2000; Maret and Li 2009). In the biological systems, various roles are performed by Zn, e.g., the catalytic (as in carboxypeptidase A and as in alkaline phosphatase), the structural (as in alcohol dehydrogenase, zinc finger), and the regulatory (as in transcription factor IIIA) (Chasapis et al. 2012). Zn is involved in various physiological processes which are essential for cellular growth, proliferation, and apoptosis (Corniola et al. 2008). The liver is an important organ for the storage and homeostasis of Zn (Stamoulis et al. 2007). Tightly controlling the intracellular concentration of Zn, i.e., pico to nanomolar levels is emerged as one of the most important mechanism employed for regulating different cellular processes (Maret 2011). Cellular zinc is stored in the cytosolic cysteine-rich protein metallothionein (MT), and homeostasis is maintained. The antioxidant properties of Zn have been attributed to induction of metallothionein (MT), GSH synthesis, regulation of oxidant production, association with cysteines, and regulation of redox signaling (Ruttkay-Nedecky et al. 2013).

Because of the diversity of cellular targets of arsenic, techniques are required that can give a holistic picture of the deleterious effects of arsenic toxicity. Estimation of biochemical parameters, hematological parameters, metabolomic profiling, and histological investigations are the various approaches that can be exploited for this purpose. Out of these, histological analysis provides the direct evidence for establishing toxicity and protective effect of the remedial substance against it (Paraguat et al. 2018). In this chapter, we are providing a detailed review regarding the application of histological investigations in identifying the trademark features of arsenic induced toxicity, and validating the protective effect of zinc in different organs and animal models.

Arsenic

Arsenic has been known to human kind since ancient times in its sulfide form. The name is derived from Greek word *Arsenikos* which means being potent. It is ranked as world's 20th most common element which exists in earth's crust, 14th in the sea, and 12th in human body. In the periodic table, arsenic belongs to group 15, i.e., Nitrogen family. It has atomic number = 33 and it possess atomic weight = 74.921 which makes it heavier than nickel, iron, and manganese but lighter than Silver, Lead, and Gold. It also exists in radioactive form as isotopes in which some are stable and some are unstable. The most stable and non-radioactive form is arsenic −75 (^{75}As) with 33 protons and 42 neutrons inside the nucleus surrounded by 33 electrons in different energy shells. It has chemical and physical properties between metal and non-metal. It has electronic configuration $1s^2\ 2s^2\ 3s^2\ 3p^6\ 3d^{10}\ 4s^2\ 4p^3$. It has greater capability to lose electrons (oxidation potential) as compared to nitrogen which increases the cationic character of arsenic. It shows four redox state(−3, 0, +3, and +5) but the valence states(−3 and 0) occur rarely in nature (Panagiotaras et al. 2012). It can occupy electrons in bonding and antibonding orbitals and exhibit properties of ligands by sharing its valence electrons (Flora 2011). It usually occurs in different forms in humans, i.e., inorganic and organic forms. The monomethylated arsenic (MMA) and inorganic arsenic are 30–300 times more toxic than the neutralized double methylated DMA, with arsenobetaine having little toxicity (Pizzorno and Crinnion 2017).

Arsenic is ubiquitously present in the earth's crust and biosphere. Human activities such as mining, use of arsenic-based pesticides and combustion of fossil fuels potentiate its accretion in the environment (Martinez et al. 2013; Singh et al. 2015; Vimercati et al. 2016; Shukla and Srivastava 2017). It is estimated that about 28,070 tons of arsenic is released from various anthropogenic sources into the environment (Pacyna and Pacyna 2001). Human exposure to arsenic compounds mainly occur either in workplaces, e.g., in smelting industries, coal fired power plants, cosmetic industries, agriculture, etc. or through arsenic contaminated food and drinking water (Chung et al. 2014).

Epidemiology of Arsenic

The utmost impact of arsenic exposure is observed to result from groundwater levels above the WHO safety standard of 10 μg/L. More than 200 million people in 70 countries are exposed to arsenic through drinking water (Hubaux et al. 2012; Hubaux et al. 2013; Bhattacharjee et al. 2013). In North American region, the United States has high risk areas such as Nevada, California, Virginia, Vermont, Maine, and New Hampshire (Banerjee et al. 2007; Hubaux et al. 2013). Many countries of Europe are also affected by arsenic contaminated groundwater like Italy, Spain, Romania, Croatia, Serbia, and Turkey (Katsoyiannis et al. 2015). The levels of arsenic in groundwater in Afghanistan [10–500 ppb] (Nriagu et al. 2007), Austrailia [1–12 ppb] (Mukherjee et al. 2006) Bangladesh [1–4730 ppb]

(Chakraborti et al. 2010), China [50–4440 ppb] (Rahman et al. 2009), Mexico [8–620 ppb] (Tuli et al. 2010), Canada [1.5–738 ppb] (Mukherjee et al. 2006) and India [10–3200 ppb] (Das et al. 2013; Srivastava and Sharma 2013) are very high.

Mechanism of Arsenic Toxicity

The mechanism of arsenic toxicity intensely depends on the species of As involved. As(V) disturbs the system of animals by interchanging phosphate in significant biochemical reactions as they have similar properties and structure (Dixon 1996). As(V) also replaces phosphate in the anion exchange in the red blood cells of humans in the transport system (Hughes et al. 2011).

Binding of As(III) with thiols is crucial to the mechanisms related to its toxicity (Hughes et al. 2011). As(III) inhibits several cellular enzymes, including pyruvate dehydrogenase, resulting in a reduced conversion of pyruvate to acetyl coenzyme A. Arsenic inhibits the uptake of glucose into cells, fatty acid oxidation, gluconeogenesis, and further production of acetyl coenzyme A. The synthesis of GSH is also inhibited by arsenic. Methylated As(III) is an effective inhibitor of GSH reductase and thioredoxin reductase (Styblo et al. 1997; Lin et al. 1999).

One important mode of action for As toxicity is the generation of reactive oxygen and nitrogen species (Lantz and Hays 2006; Kitchin and Conolly 2009). Some of the reactive species formed are H_2O_2, OH radical, reactive nitrogen species, and peroxyl radicals (Shi et al. 2004). Reactive species thus formed are responsible for various actions such as inhibition of DNA repair enzymes, genotoxicity, cell proliferation, and signal transduction (Hughes et al. 2011). The role of lipid peroxidation in As-induced oxidative stress has been extensively studied. A direct correlation can be withdrawn between plasma lipid peroxidation and arsenic levels in urine and/or tissue samples in both clinical and preclinical studies (De Vizcaya-Ruiz et al. 2009; Flora 2009; Wirtitsch et al. 2009). Arsenic-induced ROS directly attack the hydrogen atom of a methylene group adjacent to an unsaturated carbon atom (Flora 2011). Polyunsaturated fatty acids are more sensitive to free radical damage and form malondialdehyde (MDA), HNE and 2-propenal (acrolein), and isoprostanes, which can be measured in plasma and urine as indirect indicators of oxidative stress (Qian et al. 2005; Cooper et al. 2009; Wirtitsch et al. 2009). Arsenic-induced ROS is also responsible for the oxidative damage to proteins that are crucial for various cellular pathways (Flora 2011).

Use of Histology in Identifying the Hallmarks of Arsenic Toxicity

For getting an insight into the comprehensive view of a disease and its hallmarks underlying the tissue architecture, histopathology is one of the best tool. In the following sections, we have summarized the histopathological signatures that are produced due to arsenic toxicity in various organs enlisted in Table 1.

Table 1 List of various reports on histological investigations in different organs in case of Arsenic exposure

S.No	Organ	Animals Models	Doses	References
1.	Kidney	Rats	5 mg/kg, orally for 28 days Treatment of arsenic (10 mg/kg body weight of sodium arsenite for 15 days) 21 days administration of sodium arsenite (10 mg/kg, po)	ATSDR (2004) Adil et al. (2015) Momeni and Eskandari (2017) Sener et al. (2016) Mehrzadi et al. (2018) Jalaludeen et al. (2015) Yu et al. (2013) Li et al. (2010)
2.	Brain	Rats	20 mg/kg body weight-30 days 0.4 mg/kg	Dwivedi et al. (2014) Fallah et al. (2018) Li et al. (2021) Ghosh et al. (2011) Amal and Mona (2009) Sepand et al. (2016) Noman et al. (2015) Rios et al. (2009) Nageshwar et al. (2019)
		Mice	5 mg/kg	
3.	Lungs	Rats	20 mg/kg body weight orally administered- 28 consecutive days 10 mg/kg body weight orally for 4 weeks 1.57 mg/kg body weight of arsenic for 10 days 4 mg/kg body weight in drinking water	Sepand et al. (2016) Hemalatha et al. (2013) Hemmati et al. (2018) Kaushal et al. (2017) Hu et al. (2019) Kim et al. (2019)
		Mice	25 or 50 ppm of arsenic through drinking water for 20 weeks	
4.	Heart	Rats	20 mg/kg body weight orally for 28 days	Sepand et al. (2016) Hemalatha et al. (2013) Al-Forkan et al. (2016)
5.	Testis	Rats	0.5 mg/kg body weight of As2O3 for 30 days 8 mg/kg of body weight/day dose	Sharma and Kumar (2014) Jahan et al. (2016) Sarkar et al. (2008) Zeng et al. (2019) Chinoy and Jhala (2004) Momeni et al. (2012) Sharma and Kumar (2014) Manna et al. (2008) Ferreia et al. (2015) Mehrzadi et al. (2018) Guvvala et al. (2017)

(continued)

Table 1 (continued)

S.No	Organ	Animals Models	Doses	References
6.	Ovary	Black Bengal goats	4 mg/kg body weight for 7 weeks followed by 5 mg/kg body weight for next 8 weeks	Chattopadhyay and Ghosh (2010) Dávila-Esqueda et al. (2012) Wang et al. (2017) Yu et al. (2019) Maity et al. (2018) Mondal et al. (2013) Biswas et al. (2019) Ommati et al. (2020) Kumar and Sinha (2018) Islam et al. (2011)
		Mice	1.8 mg/kg body weight for 8 weeks	
		Rats	8 mg/kg body weight and 10 mg/kg body weight of sodium arsenite 0.4 ppm sodium arsenite in drinking water for 28 days	
7.	Liver	Mice	6 µg/kg body weight by daily gavages for a year	Ghatak et al. (2011) Santra et al. (2007) Messarah et al. (2012) Bodaghi-Namileh et al. (2018) Ghosh et al. (2010) Sharma et al. (2009) Zhang et al. (2013)
		Swiss albino mice	40 mg/kg body weight intraperitoneally	

Liver

It is major site for arsenic metabolism. Biomethylation of inorganic arsenic (iAs) is the main pathway for metabolism of iAs in several mammalian species, including humans (Chen et al. 2004; Drobna et al. 2010). Epidemiological studies have shown association between chronic arsenic exposure and liver disorders that include hepatomegaly, liver fibrosis, hepato-portal sclerosis, and liver cirrhosis (Das et al. 2012). Abnormal liver functions are exhibited by complex gastrointestinal issues and clinical elevations of liver functional enzymes in plasma including alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) also are associated with chronic arsenic exposure (Das et al. 2012). Ghatak et al. (2011) had evaluated the effect of long-term arsenic exposure using BALB/c mice exposed to arsenic at a 6 µg/kg body weight by daily gavages for a year. They have observed the morphological changes in liver sections stained with H&E and Sirius red that included fatty infiltration, inflammation, fibrosis, and necrosis given. In another study, high doses, i.e., 6 mg/kg body weight of arsenite were used to assess the liver histological features of hematoxylin and eosin stained liver sections on the basis of steatosis, inflammation, and necrosis. They reported that hepatic steatosis was macro vesicular in nature and there is accumulation of lipid droplets in the hepatocytes (Santra et al. 2007). A dose of 5.55 mg/kg b.w/day of $NaAsO_2$ given intraperitoneally 3 consecutive weeks is able to produce pathological alterations such as cytoplasmic vacuolization and the occurrence of cellular wreckages within the central vein (Messarah et al. 2012). Similar results were reported by Bodaghi-Namileh

et al. 2018. Ghosh et al. (2010) have observed the few focal areas of necrosis, hyperplasia of Kupffer cells, and fibrosis in the periportal region after a single exposure of $NaAsO_2$ (13 mg/kg body weight). While karyorrhexis, karyolysis, cytoplasmic vacuolization, and centrilobular necrosis were documented in the liver sections of Swiss albino mice injected with 40 mg/kg body weight intraperitoneally (i.p.) (Sharma et al. 2009). Arsenic given in the form of arsenic trioxide had also produced histological alterations such as hepatocyte vacuolization, inflammatory cells infiltration, disorganization of parenchyma, necrosis, and dilatation of inter-hepatocellular spaces (Zhang et al. 2013).

Kidney

It is major excretory organ. It is reported that chronic arsenic exposure can leads to development of kidney cancer, whereas its acute intoxication can cause tubulointerstitial nephritis following fluid imbalances. However, the kidney is less sensitive to chronic arsenic than most other organ system (ATSDR 2004). Histology provides a successful tool in studying the morphological changes in arsenic-induced nephrotoxicity. Adil et al. (2015) had demonstrated the deteriorating effects of sodium arsenite at a dose of 5 mg/kg, orally for 28 days in rats. The hematoxylin and eosin-stained kidney sections revealed the presence of intrinsic lesions within the glomeruli and epithelium. Additionally, it also showed the presence of edema, inflammatory infiltration, and increased glomerulus basement membrane thickness. In another study, similar histopathological changes were induced by sodium arsenite (same) were observed in the kidney tissue when given intraperitoneally (Momeni and Eskandari 2017). Sener et al. (2016) had observed that the treatment of arsenic (10 mg/kg body weight of sodium arsenite for 15 days) was able to deteriorate the structure of the kidney tissues, with a remarkable degeneration and dilation occurred in the tubules. They had also monitored that some tubules cells were deformed and switched to squamous cells. Also, the proximal cells had lost their density and discharged into the lumen and the brush border cells present on the tubule walls were deformed (Sener et al. 2016). Similar result were reported by Mehrzadi et al. (2018) who demonstrated that 21 days administration of sodium arsenite (10 mg/kg b.w) through drinking water has induced extensive injuries in kidney tissues validated by the presence of glomerular hypertrophy, congestion, leukocyte infiltration, edema, and necrosis in kidney sections. These observations are further supported by other studies (Jalaludeen et al. 2015).

Arsenic given in the form arsenic trioxide was also capable of inducing renal injury. Yu et al. (2013) had demonstrated the As_2O_3-induced histopathological changes in the renal tissues of cats such as the epithelial atrophy, destruction of tubular structure, inflammatory cell infiltration, dilation and hyperemia of glomerular capillaries. In another study, Li et al. (2010) had observed the effect of As_2O_3 on the renal sections of mice kidney. Cellular swelling, tubular dilation, and lymphocytic infiltrations were observed. Additionally, loss of cell to cell contacts and loss of brush border were shown in the epitheliums of proximal convoluted tubules (Li et al. 2010).

Brain

It is one of the major target organ for arsenic toxicity. Brain is predominantly vulnerable to oxidative damage due to high consumption of inspired oxygen. In a variety of studies, histology has been successfully used to understand the toxic effects of arsenic in the brain tissue. Dwivedi et al. (2014) had shown that the exposure of 50 ppm sodium arsenite in drinking water was able to produce gliosis, mild to moderate neuronal vacuolization, shrunken pyknotic neurons along with chromatolysis of nuclear material and edema. Severe white matter lesions and cellular damages are seen in the hippocampal CA1 areas of rats exposed to single dose of arsenic (20 mg/kg body weight) every other day for 30 days (Fallah et al. 2018). In the study of Li et al. (2021), degenerated neurons, cytological alterations, cell shrinkage, and clustering of nerve cells were observed in the histological sections of mice brain exposed to 5 mg/kg body weight of As in the form of $NaAsO_2$. These observations were also reported by other studies (Ghosh et al. 2011; Amal and Mona 2009; Das et al. 2010; Sepand et al. 2016). In another study, edema, intracellular spaces, edematous changes have been seen (Noman et al. 2015).

Rios et al. (2009) have analyzed nitrergic markers and the morphology of the striatum of rats exposed to arsenite at 3 ppm in drinking water, resulting in an approximate daily dose of 0.4 mg/kg, from gestation, lactation and throughout the development until adulthood. They have examined the fiber tracts by means of the Klüver–Barrera, Bielschowsky and toluidine stains and reported the appearance of revealed discontinued axons, coexistence of thin and thick axons, and undulated fibers. Severe damage was observed in the neurons only in arsenic-exposed animals. The fiber tracts perceived in toluidine-stained sections demonstrated irregular transversal section with heterogeneous size, thickness, and even discontinuity of the myelin sheath (Rios et al. 2009). Nageshwar et al. (2019) have studied the effect of arsenic toxicity when given in the pentavalent form, i.e., sodium arsenate (100 ppm/kg b.w) through drinking water and reported the neural cells with irregular shape and cyton, axon, and dendrite were degenerated in the histological micrographs of brain sections.

Lungs

Sepand et al. (2016) have reported the accumulation of lipofuscin pigment in the pulmonary tissue histological sections of rats that received sodium arsenite (20 mg/kg body weight orally administered for 28 consecutive days) in comparison with control rats. In another study, a dose of 10 mg/kg body weight orally for 4 weeks is able to produce congestion, emphysematous, and ruptured alveoli in the lungs tissue of rats as revealed by the analysis of H and E stained sections (Hemalatha et al. 2013). In similar lines, Hemmati et al. (2018) have documented the alveolar wall degeneration, pulmonary edema, and inflammation in the lung tissue sections of mice being exposed to 25 or 50 ppm of arsenic through drinking water for 20 weeks While the intraperitoneal dose of 1.57 mg/kg body weight of arsenic for 10 days initiated severe histological modifications in lung tissue disclosing bronchiolar

epithelium degeneration, elevation in the number of alveolar macrophage, alveolar space reduction, and conjunction in alveolar duct and alveolar hyperplasia (Kaushal et al. 2017). Also, arsenic administration in the form of As_2O_3 (4 mg/kg body weight in drinking water) lead to the widening of alveolar septa with capillary congestion and inflammatory cell invasion (Hu et al. 2019). Histological analysis also provides evidence in support of the role of arsenic as a Pro-Metastatic Carcinogen in case of intravenously injected melanoma cells into lung (Kim et al. 2019).

Heart

It is another internal organ affected by arsenic toxicity. Inflammation has been found to be one of the most important factors that contribute to cardiovascular disease (Das et al. 2012). Mild myocardial necrosis was reported in $NaAsO_2$(20 mg/kg body weight orally for 28 days) treated animals as compared with the control group (Sepand et al. 2016). Hemalatha et al. (2013) have observed hemorrhages in myocardium, separation, and degeneration of muscle bundles in the H and E stained sections of heart after As exposure. While Al-Forkan et al. (2016) have also documented similar results.

Testis

Testis is one of the sensitive organ because of its cell renewal system. The deleterious effects generated in this tissue may pass through generation and also effect the progeny (Sharma and Kumar 2014). A variety of doses, duration of exposure, and form of As were reported to induce alteration in male reproductive functions (Jahan et al. 2016; Sarkar et al. 2008; Zeng et al. 2019). This fact is well substantiated by the histological findings. Histological examination of the experimental testis revealed that the treatment of 0.5 mg/kg body weight of As_2O_3 for 30 days resulted in the apical degradation and convergence of tubules, destruction of germinal epithelial cells, and absence of sperm in the lumen (Chinoy and Jhala 2004). Additionally, the Leydig cells were not also clearly visible (Chinoy and Jhala 2004). In another study, the exposure of 8 mg/kg of body weight/day dose of sodium arsenite was able to produce remarkable changes in the testicular tissue including reduction in the height of seminiferous tubule's wall and mean diameter of seminiferous tubules whereas increase in the seminiferous tubule's lumen as compared to control groups were observed (Momeni et al. 2012). In the study of Sharma and Kumar (2014) also, the shrinkage of tubules and reduction in the population of germ cells was apparent on As exposure. Similar manifestations were also observed in the studies of Manna et al. (2008), Souza et al. (2015) and Mehrzadi et al. (2018) with slightly higher doses.

Souza et al. (2015) and Guvvala et al. (2017) have seen the effect of different doses of arsenate exposure on the testicular tissue. The analysis of testicular sections revealed that arsenate exposure was also leading to abnormal organization of germinal epithelial cell layer of seminiferous tubules with decrease in the number of spermatozoa in the lumen. Therefore, it can be suggested that arsenic-induced

oxidative stress might cause disintegration of the epithelial cells and alteration in sperm production as evident from the histological outcomes of arsenic toxicity.

Ovary

Being the main reproductive organ in females and having higher cellular turnover rate, ovaries are very susceptible to oxidative stress. As we knew that arsenic is a potent inducer of oxidative stress. Therefore, it can produce deleterious effects in the ovarian cells that can be examined by using histological investigations. The studies mentioned in this section are able to validate this fact. A dose of 0.4 ppm sodium arsenite in drinking water for 28 days is potent enough to produce a reduction in the numbers of primary classes of preantral, antral, and graafian follicles, along with an elevation in the number of regressing follicles (Chattopadhyay and Ghosh 2010). Uterine endometrium, myometrium, and epithelial layer degenerations were described by a significant thinning of these layers arsenic intoxicated rats (Chattopadhyay and Ghosh 2010). Dávila-Esqueda et al. (2012) have seen the effect of sodium arsenite at 3 ppm in drinking water on female rats from mating, gestation, and after the birth (the female offspring continued As exposure via lactation). Microscopic examination of ovarian tissue demonstrated a pyknotic chromatin, cariorexis, and substantial cytoplasmic vacuolization after As intoxication that in few cells the nucleus has been pushed to the cell periphery, giving an appearance of signet ring cells. Additionally, stroma vasculature was also damaged (Dávila-Esqueda et al. 2012). According to HE staining of slices of the rat ovarian tissue, the doses of 8 mg/kg body weight and 10 mg/kg body weight of sodium arsenite can lead to distinctive reduction in the number of primary and antral follicles and the destruction of ovarian tissue validated by the appearance of few structurally complete follicles and abundant atretic follicles (Wang et al. 2017; Yu et al. 2019; Maity et al. 2018). In other studies on the rat model, arsenic was given in the form of arsenic trioxide and similar histo-architectural defects were documented (Mondal et al. 2013; Biswas et al. 2019; Ommati et al. 2020).

Using histopathological investigations, Kumar and Sinha (2018) have seen the effect of arsenic on mice model at the dose of 1.8 mg/kg body weight for 8 weeks by gavage method. They have reported the degeneration in the ovarian follicles and cytoplasmic and nucleoli vacuolization was witnessed in corpus luteum along with ruptured germinal epithelial lining. Likewise, Islam et al. (2011) have studied the effect of sodium arsenite (4 mg/kg body weight for 7 weeks followed by 5 mg/kg body weight for next 8 weeks) on black Bengal goats. In the histopathological analysis using Goldner's Trichome stain, ovarian follicular degeneration was detected.

Approaches to Combat Arsenic Toxicity

Arsenic exerts its toxic effects majorly either by the depletion of antioxidants defenses and production of ROS or by interacting with the thiol moiety of proteins. Therefore, the therapeutic approaches develop to combat arsenic toxicity are either based on strategy to sequester arsenic with the help of thiol containing chelators or

increase the antioxidant capacity of cells. Following section explains the strategies used to combat arsenic toxicity based above mentioned points:

Chelation Therapy

Chelating agents are organic compounds capable of linking with metal ions to form a complex ring-like structures called chelates. The chelation prevents arsenic from interacting with biological targets such as proteins and DNA. Moreover, it helps in the elimination of arsenic from the body (Flora et al. 2007). These agents should bear some important characteristics like non-toxic, lipid soluble, bear more than one binding site, and also possess high affinity toward arsenic and gets eliminated from body in the complexed state (Flora et al. 2010; Clarke et al. 2012). Brtish anti-lewisite (BAL) is a dithioglycerol developed by British to counteract Lewisite (β-chlorovinyldichloroarsine) during World War I. BAL could sequester As, preventing its binding to proteins and protect them. Development of lipophilic, nontoxic chelators is the new era for the treatment of arsenic poisoning. Most commonly used chelators are Sodium 2,3-dimercaptopropane 1-sulfonate (DMPS), Dimercaptosuccinic acid (DMSA), and one of its analogues, monoisoamyl-DMSA (MiADMSA) (Flora and Pachauri 2010). Monotherapy with chelators may not be beneficial in providing better clinical recoveries. Thus combination therapy utilizing antioxidants such as N acetylcysteine NAC, taurine, or herbal extracts with a thiol-chelating agent will be beneficial. Use of two structurally different chelating agents, too, can be a useful strategy (Flora 2011).

Use of Nutritional Supplements as Antioxidants

The antioxidants can be supplemented for reducing oxidative stress. Antioxidant molecules play a crucial role in countering free radical induced damage to macromolecules and has found to heal the free radical mediated cell damage. The antioxidants can be phenolic and non-phenolic. The phenolic compounds can be subclassified as Flavonoids, Stilbene, Phenolic acids, and Lignins (Manach et al. 2004). Examples of phenolic antioxidants are Biochanin A, Curcumin, Diallyltrisulphide, Eugenol, Ellagic acid, Epigallocatechin-3-gallate, Naringin, Phloretin, Quercetin, Resveratrol, and many more. Examples of non-phenolic compounds α-Lipoic acid, α-Tocopherol, Flax seed oil, Morphine, Omega–3-fatty acid, Taurine, Vitamin C, and Vitamin E (Pace et al. 2017) Many of these antioxidants have shown promising results in order to ameliorate toxic effects of arsenic in different tissues by restoring the antioxidative defenses of cells (Pace et al. 2017). Although, their mechanism of action may differ.

Because of the antioxidant properties of selenium and zinc, these essential metals may play a protective role in case of arsenic toxicity. The low concentration of selenium provided protection against arsenic toxicity by forming an As–Se compound $[(GS3)2AsSe]^{-}$ that gets excreted from the body. But, at higher concentration

selenium produced synergistic toxic effects with arsenic by reacting with S-Adenosyl methionine, glutathione, and modification of arsenite methyltransferase (Sun et al. 2014). The antioxidant and detoxifying effects of selenium against arsenic toxicity in the *Poeciliopsis lucida* hepatocellular carcinoma cell line 1 (PLHC-1) were reported (Selvaraj et al. 2012). The eliminaton of methylated arsenic in urine of selenium deficient mice is slow (Kenyon et al. 1997). Arsenic and selenium containing compound named as selenobis (S-glutathionyl) arsinium ion $[(GS)_2AsSe]^-$ can be readily excreted from hepatocytes in bile (Gailer et al. 2000; Ponomarenko et al. 2017). Another mechanism behind the protective role of selenium against arsenic induced oxidative damage is the upregulation of selenoproteins like GPx and thioredoxin reductases (Rahman et al. 2018). The role of zinc in providing protection against arsenic toxicity is discussed in later sections.

Zinc

Zn is found in group IIb of the periodic table, which also contains toxic metals cadmium and mercury. Zinc independently isn't redox active and it does not interact directly with ROS or carbon centered free radicals. Its antioxidant properties have been attributed to induction of metallothionein (MT), GSH synthesis, regulation of oxidant production, association with cysteines, and regulation of redox signaling (Ruttkay-Nedecky et al. 2013) (Table 2).

Tightly controlling the intracellular concentration of Zn, i.e., pico to nanomolar levels is emerged as one of the most important mechanism employed for regulating different cellular processes. The mechanism of zinc efflux and uptake, sequestration, and release across the biological membranes is well documented. There are two classes of zinc transporters that are mainly involved in zinc transportation, i.e., ZnT (Slc30a family proteins) and ZIP (Slc39a family proteins) transporters. There are

Table 2 Histological studies on the ameliorating effect of Zinc in combating arsenic toxicity

S.No	Organ	Animals Models	Doses	References
1.	Liver	Rat	Zn (227 ppm in drinking water) and As (100 ppm drinking water) for 3 months	Kumar et al. (2010)
2.	Liver Kidney	Rat	Zn (227 ppm drinking in water) against As (37 ppm in drinking water) for 12 weeks	Garla et al. (2021)
3.	Liver	Rat	Zn (153 μmol/kg subcutaneously) and As (75 μmol/kg subcutaneously) for 2 days	Ganger et al. (2016)
4.	Kidney	Rat pups	Zn (20 mg/kg body weight) in case of As (5 mg/kg body weight)	Ghabaee et al. (2017)
5.	Spleen	Common carp	Zn (1 mg/L) and As (2.83 mg/L)	Wang et al. (2021)
6.	Intestine	Common carp	Zn (1 mg/L) and As (2.83 mg/L)	Zhao et al. (2019b)

9 ZnT and 14 ZIP transporters (Fukada and Kambe 2011). The ZnT facilitate the efflux of zinc out of cells (Palmiter and Huang 2004), whereas ZIP facilitate influx of zinc inside the cells (Eide 2004). Sequestration of Zn ions in a cellular compartment is one way of storing Zn ions in the cell and then releasing them again. This compartmentalization is a central aspect of the cellular homeostatic control of Zn and requires Zn transporters and MTs (Maret 2011).

Histological Studies on the Ameliorating Effect of Zinc on Arsenic Toxicity

The application of histology is not restricted in establishing the structural trademarks of any disease but also in providing confirmation for the remedial effects of any nutritional supplements. For studying the ameliorating effects of zinc in case of chronic toxicity also, histological analysis has been frequently used as summerized in Table 2. Kumar et al. (2010) have investigated the role of Zn (227 ppm drinking water) against As (100 ppm drinking water) toxicity in rat liver. The zinc supplementation considerably improves histoarchitecture of liver tissue, although few regions of liver sections were still showing the signs of degeneration and necrosis. Similar results were reported by Garla et al. 2021. In another study, Ganger et al. (2016) have documented the betterment in the histological features of liver tissue after Zn treatment in case of acute As toxicity. Protective role of Zn (20 mg/kg body weight) in case of As (5 mg/kg body weight) toxicity in the kidney tissues of rat pups during gestation and lactation was also reported (Ghabaee et al. 2017). The histopathological examination of PAS stained kidney sections revealed the swelling in the renal tubular epithelial cell, damage in brush border, pyknotic nuclei of renal epithelium, cellular degeneration and necrosis, whereas, Zn supplementation ameliorated these histopathological changes significantly. Furthermore, Wang et al. 2021 have reported that Zn offers protective potential in combating against As induced splenic damage in common carp and the histopathologic changes were improved. Zhao et al. (2019a) have seen the effect of Zn treatment (1 mg/L) along with arsenic trioxide (2.83 mg/L) for a month on the different intestinal regions of common carp. The histopathological damages include swelling and increasing number of neutrophil infiltration, which was then ameliorated when Zn was co-administrated.

Applications of Histology in Other Diseases

In this chapter, we have reviewed the use of histology for studying the toxicity of arsenic and ameliorating effect of zinc, a nutritional supplement having antioxidant properties. Zinc being a co-factor of a number of enzymes, plays a crucial role in various cellular processes. Zn supplementation has shown to play role in immune functions, stress response, improved cognitive functions, and age-related neurodegenerative disorders (Mocchegiani et al. 2011). There are many other disorders such as cancer, viral infections, where the studies on the function of Zn are far from adequacy

and histology can play a major part in that. As we know, arsenic induced oxidative stress is major mechanism for its deleterious effects. Likewise, Zn histological can be employed for identifying the potential other nutritional antioxidants against arsenic toxicity. Moreover, histology can also be used for understanding the mechanism of other heavy metals (like Pb, Cd, Hg etc.) and environmental pollutants.

Mini-Dictionary of Terms

- Sequestration: To seclude or put aside a substance from other things.
- Chelators: A binding substances that can suppresses the biological activity by forming chelates.
- Karyorrhexis: Fragmentation of nucleus and its chromatin.
- Vacuolization: Formation of vacuole like structures within the tissue.
- Dilation: The state of enlarging or distending.
- Parenchyma: The functional tissue of any organ distinctive from its surrounding connective tissue and circulatory vessels.

Key Facts About Arsenic Toxicity

- More than 230 million people across the globe are at the risk of arsenic exposure.
- Over the last decade, the number of countries that were reported to have elevated levels of As in groundwater rises from 40 to 107.
- Toxicity of arsenic compounds is highly dependent upon their oxidation state and chemical composition.
- Arsenic imparts its toxicity by interacting with the sulfhydryl groups of proteins, generation of ROS and replacing phosphate.
- Chronic arsenic exposure of inorganic arsenic through drinking water causes skin lesions, hyperkeratosis, cancers, blackfoot disease, vascular diseases, and diabetes.

Summary Points

- The diversity of cellular targets for arsenic binding adds complexity in understanding its toxicity.
- For identifying the tissue architectural hallmarks related to arsenic toxicity, histology is one of the best tool.
- Histology has been extensively used for characterization of trademarks of deleterious effects produced by arsenic exposure in different organs.
- Zinc, a nutritional supplement, having the antioxidant properties and ability to induce metallothionein, has been studied for its protective value against arsenic.
- Histology provides a direct evidence for establishing the ameliorating role of Zn against As toxicity.

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Measures of Iron Metabolism and Applications to Dietary Patterns

11

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Abstract

Iron deficiency (ID) remains the most common nutritional disorder worldwide. Although iron is biologically essential, tight regulation of iron balance is critical to prevent iron toxicity. Significant progress has been made on iron disorder diseases since the discovery of hepcidin in 2000. Hepcidin, a master iron regulator, controls iron balance through degradation of ferroportin, the only known mammalian iron exporter. Insufficient hepcidin synthesis is central to hereditary iron overload. Uncontrolled inflammation promotes hepcidin overproduction and triggers functional ID and anemia of inflammation. Hence, the etiology of iron restriction and iron overload is distinct from absolute ID. This chapter provides a comprehensive overview of diet and iron disorder with special focus on absolute ID, functional ID, and hereditary iron overload. Advances in the understanding of hepcidin and its interactions with diet may help translate into making effective dietary approaches for patients with different types of iron disorder.

Keywords

Iron metabolism · Hepcidin · Dietary approaches · Absolute iron deficiency · Functional iron deficiency · Anemia of inflammation · Iron overload

Abbreviations

ACD	Anemia of chronic disease
AI	Anemia of inflammation
CI	Confidence interval
Cp	Ceruloplasmin
CRP	C-reactive protein
CT	Computerized tomography
DII	Dietary inflammatory index
DMT1	Divalent transporter 1
EPO	Erythropoietin
GDF 15	Growth/differentiation factor-15
Hb	Hemoglobin
HFE	Hemochromatosis
HH	Hereditary hemochromatosis
HJV	Hemojuvelin BMP co-receptor
ID	Iron deficiency
IDA	Iron deficiency anemia
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MRI	Magnetic resonance imaging
Mφ	Macrophages
ORs	Odds ratio
PUFA	Polyunsaturated fatty acid
RBCs	Red blood cells

RDAs	Recommended daily allowances
SCA	Sickle cell anemia
sTfR	Soluble transferrin receptor
TfR2	Transferrin receptor 2
TIBC	Total iron-binding capacity
Tsat	Transferrin saturation
Vit	Vitamin
WHO	World Health Organization
WMD	Weighted mean differences
ZnPP	Zinc protoporphyrin

Introduction

Overview of Iron Metabolism and Iron Disorder Disease

Iron Distribution

Human adults contain approximately 3–4 g iron in the body (Fig. 1a). About 60–70% of the body's iron is stored in the Hb (Hb) of red blood cells (RBCs) (1800 mg), erythroblasts in the bone marrow (300 mg), or myoglobin in muscle tissue. The other third is found in tissue macrophages (600 mg), liver (1000 mg), heart, and skeletal muscle tissues (300 mg). Transferrin is the main iron carrier which transports iron throughout the blood to the body. Although <0.1% (3–4 mg) of iron is found in the plasma iron pool, turnover is high in order to support the iron demand of erythropoiesis (20–25 mg per day) and other iron-demanding organs. Absorption of iron is tightly regulated since there is no iron excretion route once it enters the body. About 1–2 mg of iron is absorbed from a typical mixed diet (15–20 mg/day), which is only to compensate for the basal iron losses (<1 mg/day). Daily iron required for erythropoiesis (<30 mg) is predominantly derived from recycling of heme iron from senescent RBCs by macrophages, which supply ~95% of daily iron requirement.

Hepcidin-Ferroportin Axis and Iron Disorder Disease

Significant progress has been made in our understanding of iron disorder diseases since the discovery of hepcidin (HAMP) in 2000 (Krause et al. 2000). Alteration of the hepcidin-FPN (FPN) axis explains most iron disorder diseases (Fig. 1b). Hepcidin, mainly produced by the liver, is the key hormone that controls iron balance (Ganz and Nemeth 2011). By limiting the expression of the only known iron exporter, FPN, hepcidin controls systemic iron levels by decreasing dietary iron absorption and inhibiting the release of stored iron from the liver and macrophages. Absolute iron deficiency (ID), referring to true ID with low iron stores, tends to occur in malnourished people with low dietary iron intake. Serum hepcidin decreases when blood iron level is low, thus enabling increased absorption of dietary iron through duodenal FPN. A kidney-derived erythropoiesis mediator, erythropoietin (EPO), also promotes iron absorption via suppressing hepcidin synthesis. Functional ID,

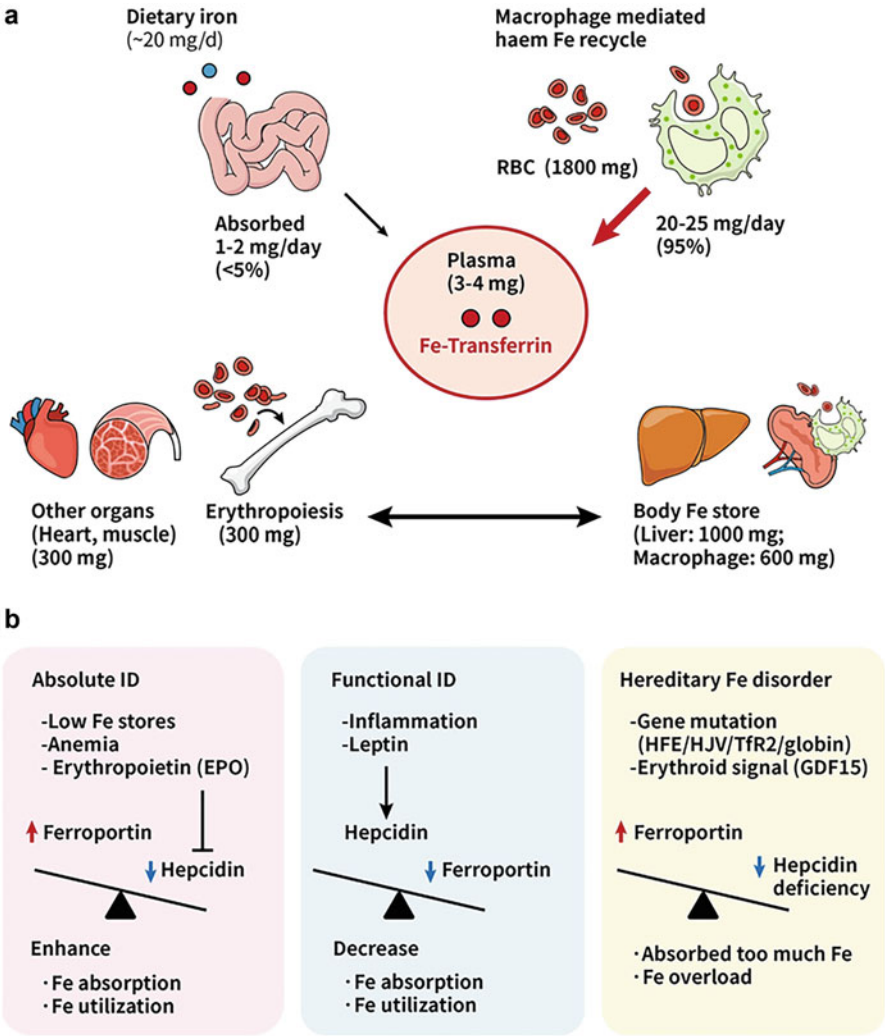

Fig. 1 Overview of iron homeostasis (**a**) and iron disorder diseases (**b**)

frequently observed in patients with obesity or chronic disease, is characterized by insufficient mobilization of iron from iron stores to the circulation. Inflammation and leptin trigger hepcidin over-synthesis. Elevated hepcidin then decreases iron absorption, therefore less mobilization of body iron stores. Functional ID may progress to anemia of inflammation (AI) when circulating iron levels are unable to meet the demand of bone marrow erythropoiesis. Hereditary iron disorders are genetic disorders that cause the body to absorb too much iron from the diet due to ineffective erythropoiesis (e.g., ß-thalassemia, sickle cell anemia) or mutation of hemochromatosis (HFE) genes (e.g., HFE, HJV, TfR2) that causes hepcidin deficiency. Absent or

low serum hepcidin levels lead to uncontrolled iron absorption and promote the risk of iron overload.

Iron Requirement

Iron balance refers to a steady state where iron intake equals iron requirements. The iron absorbed from the diet must cover both the iron losses from the body and extra requirements, such as increased erythropoiesis during growth or pregnancy. The amount of iron required for growth ranges from ~0.2 to 0.6 mg/day for children and adolescents and ~0.8–7.5 mg iron/day for the early and late pregnancy (Hallberg et al. 2000). Iron losses include a loss of <1 (~0.6–1) mg iron/day from the mucosal surfaces (e.g., skin, gut, urinary tract, and lung), as well as menstrual blood iron losses (~0.5–1.9 mg iron/day) for reproductive-age women and teenagers. Hence, the daily iron requirement for adult men and postmenopausal women is estimated to be ~1 mg iron/day. For women of reproductive age, about 1.5–3 mg/day of iron must be absorbed from the diet to replace the basal and menstrual losses. In gestation, it is suggested that ~4.4 mg iron/day must be absorbed from the diet, with 0.8 mg iron/day required for the first trimester, steadily increasing to 7.5 mg iron/day in the last trimester of pregnancy (Milman et al. 2017). Because breast milk contains very low levels of iron (0.3–0.6 mg Fe/L), term infants are born with ~250–300 mg iron to support the growth demand of the first 6 months of life. Therefore, infants are more likely to develop absolute ID or iron deficiency anemia (IDA) if their mothers are iron depleted during gestational period (Table 1).

Recommended Daily Allowances

Table 2 shows the recommended daily allowances (RDAs) of iron intake. It varies according to age, gender, pregnancy, as well as country and region. RDAs indicate that the daily amount of iron consumed from the diet is sufficient to meet the needs of

Table 1 Requirement of daily absorbed iron (mg/day) to support the growth, basal losses, and menstrual losses throughout human life (WHO 2004; Milman 2006)

Group	Age	Male	Female
Infants	0.5–12 months	0.7–0.9	
Children	1–10 years	0.5–0.9	
	11–17 years	1.2–1.9	1.2–3.3
	>18 years	1.1–1.4	1.5–2.9
Postmenopausal	>50 years	1.1–1.4	0.9–1.1
Pregnancy			0.9–7.5[d]
Lactating			1.2–1.5

[a]Total absolute requirements = requirement for growth + basal losses + menstrual losses

[b]Range indicates mediam~95th percentile of daily absorbed iron (mg/d)

[c]Adapted from the World Health Organization report (2004) (WHO 2004)

[d]Adapted from a review article by Nils Milman (Milman 2006)

Table 2 Summary of recommended daily iron (RDA) intakes (mg/day)

Age groups	Age	Male	Female
Infants	7–10 months	7.8–11	
Children	1–9 years	5–10	
Adolescents	10–18 years	8–24	
Reproductive age	19–50 years	8–11.9	15–26
	>50 years	8–11.5	
Pregnancy			+9–38
Lactation			+9–30

97–98% of individuals to prevent absolute ID or IDA in a specific life-stage groups (Milman et al. 2017). Developing countries (e.g., Vietnam, African nations) tend to have the highest RDAs of iron for adolescents and reproductive-age women. Indonesia (+9 mg/d) has the lowest and Africa the highest (38 mg/d) RDAs of iron in pregnancy. This results in two- to four-fold regional differences in RDAs of iron.

Iron Bioavailability in Foods

Broadly speaking, iron absorption is regulated by both external (e.g., dietary factors) and internal (e.g., body iron stores, inflammation, genetic factors, and growth demands) factors. For healthy children and adults, iron bioavailability mainly depends on dietary factors, growth demands, and body iron levels. Iron utilization in disease or the elderly is more likely to be influenced by inflammation, infection, and chronic diseases.

Types of Iron Sources in Food

Dietary factors are the major external factors that influence iron bioavailability. There are two types of iron sources in food: heme and non-heme iron. Heme iron is mainly found in animal products (e.g., meat, poultry, livestock, fish) and non-heme iron in both plant-based and animal-derived foods. Heme iron is better absorbed (~25%) than non-heme iron (~17%). For meat-eating (mixed diet) individuals, heme iron accounts for <10% of the daily iron intake, but it contributes to about 40% of the dietary absorbed iron (WHO 2004). The amount of absorbed iron in a typical mixed diet or vegetarian diet is approximately 14–18% (Hurrell and Egli 2010) or 5–10% (Saunders et al. 2013), respectively. About 90% of dietary iron is derived from plant-based non-heme iron. However, only ~10% of non-heme iron is absorbed from staple foods such as rice (11%) or steamed buns (8%) (Yang et al. 2016).

Factors That Affect Dietary Iron Absorption

Although non-heme iron is the main source of dietary iron, bioavailability of non-heme iron is strongly influenced by the presence of iron enhancers or inhibitors in meals. Heme iron is well absorbed and less affected by other dietary factors. Table 3 summarizes factors that affect iron absorption (Table 3).

Table 3 Factors that affect iron absorption

B	B	B
Diet	MFP factors	Vegetarian-related eating habit (e.g., vegan diet)
	Ascorbic acids (vitamin C)	Low intakes of meat, fish, and poultry
	Acids (gastric acids, citric acids)	Phytate and oxalic acid in vegetables, grains, and legumes
	Copper	Polyphenols (e.g., tannic acid, quercetin) in tea, coffee, wine, vegetables, fruits
	ß-Carotene	Sugars (fructose and sucrose)
		Copper deficiency or metal overload
		Vitamin A deficiency
		Protein derived from milk, egg, soy beans
		Energy and protein deficiency
Fe status	Iron deficiency/anemia	High body iron store
	Growth demands	
	Iron loss (e.g., menstruation, bleeding, blood donation)	
Disease and gene	Hereditary iron disorder (e.g., primary hemochromatosis, thalassemia, sickle cell anemia)	Inflammation (e.g., obesity, chronic kidney disease) Drugs (antacids, gastric acid inhibitors, nonsteroidal anti-inflammatory drugs)

Iron Enhancers

MFP Factors

Meat, poultry, fish, and shellfish contain both heme and non-heme iron. Meat and organs (e.g., liver) are good sources of heme iron but also contain peptides, known as MFP factors, that promote absorption of non-heme iron from other foods. Iron-binding low-molecular-weight peptides or phospholipids (e.g., cysteine or histidine-containing peptides, L-alpha-glycerophosphocholine, glycosaminoglycans) are released during digestion of muscle tissue and bind iron in a soluble form in the stomach (Lesjak and Srai 2019; Milman 2020). This prevents the chelation of non-heme iron by dietary inhibitors (e.g., phytic acid and polyphenols). The iron-peptide complex, a soluble and more absorbable form, across the apical surface of enterocyte in duodenum possibly through peptide transporter, and not divalent transporter 1 (DMT1), enters the bloodstream through iron exporter FPN.

Organic Acids (Vitamin C, Citric Acids, Malic Acids) and Gastric Acids

Vitamin C (Vit C) (also known as ascorbic acid) is a powerful antioxidant and the most potent enhancer of non-heme iron absorption (Collings et al. 2013). Vit C can prevent the formation of insoluble iron with phytate or polyphenols (e.g., tannins). By acting as an electron donor, Vit C can reduce ferric iron (Fe^{3+}) to soluble ferrous iron (Fe^{2+}), thus promoting non-heme iron absorption. The addition of Vit C to a single test meal (Vit C/Fe molar ratio ~ 2:1) promotes non-heme iron bioavailability. Other organic acids such as citric acids or malic acids in fruits as well as gastric acids

in stomach also promote non-heme iron absorption, possibly through the same mechanism. Patients with hypochlorhydria (low stomach gastric acid) or those regularly using antacids or proton pump inhibitors to treat gastroesophageal reflux diseases or peptic ulcers are at risk of ID.

Alcohol

Alcoholic beverages with low concentrations of ethanol are potent inducers of gastric acids which aid non-heme iron absorption. Ethanol may also regulate iron bioavailability via regulating the hepcidin-FPN axis (Milman 2020). Ethanol decreases hepatic hepcidin synthesis, leading to high duodenum levels of iron exporter FPN, thereby promoting iron absorption in the gut.

Copper

Ceruloplasmin (Cp), a copper-containing enzyme ferroxidase, promotes the release of absorbed iron to the bloodstream. Cp oxidizes intracellular ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) and loads ferric iron onto transferrin (Harder et al. 2020). Transferrin then delivers iron to various tissues and organs in the body.

ß-Carotene and Vitamin A

β-Carotene may also enhance non-heme iron absorption from plant-based foods. Vit A (rice, two-fold; wheat, 0.8-fold; corn, 1.4-fold) and β-carotene (rice, three-fold; wheat, 1.8-fold; corn, 1.8-fold) increase non-heme iron absorption in a cereal-based diet (Garcia-Casal et al. 1998). β-Carotene may enhance non-heme iron uptake through ferric iron-solubilizing effects by chelating iron inhibitors (phytate or polyphenols) in foods.

Body Iron Stores

Iron absorption increases when body iron store is depleted, during increased erythropoiesis in pregnant women and children, and after blood loss. Contrarily, iron absorption decreases when iron stores are high. A great variation in the amount of iron absorbed during pregnancy is reported: 7%, 36%, and 59% for 12, 24, and 36 weeks of gestation, respectively (Whittaker et al. 2001). On average, iron absorption increased by 17% (range 19–21%) during gestation after accounting for the absorbed iron incorporated to the fetus (10%; range 19–21%) and for iron retained within the placenta (7%, range 39–42%)(Delaney et al. 2020).

Iron Inhibitors

Major iron inhibitors include (1) plant-derived components: phytates in cereals and legumes, oxalic acids in green leafy vegetables, and polyphenols in tea, coffee, wine, fruits, and vegetables; (2) iron inhibitory proteins found in legumes (e.g., soy protein), eggs, or milk; and (3) divalent metals (e.g., calcium).

Phytate and Oxalate

The inhibitory effects of plant-derived foods on non-heme iron absorption are largely attributed to phytate, polyphenols, and to a lesser extent, oxalic acid (Lesjak

and Srai 2019; Milman 2020). Phytate (phytic acid) is the main storage compound of phosphorus in grains, legumes, or nuts and cannot be destroyed in human gastrointestinal tract. Phytate decreases non-heme iron absorption via forming a complex with ferric iron, possibly binding through the phosphate ester group (Lesjak and Srai 2019). Phytate in cereals or legumes can be removed by food preparation methods such as milling, soaking, germination, and fermentation (Lesjak and Srai 2019; Milman 2020). The addition of Vit C in the meal can also prevent the iron-binding effects of phytate (Lesjak and Srai 2019; Milman 2020). Oxalate (oxalic acids) is naturally occurring in plant-based foods and is present in high amounts in green leafy vegetables. Oxalate can also decrease calcium absorption by forming a complex of calcium oxalate. It is suggested that iron suppressing effects of oxalate may be due to the concomitant presence of other iron inhibitors (e.g., calcium, phytate, polyphenols) in vegetables or legumes (Milman 2020).

Polyphenols: Tea and Coffee

In recent years, the iron regulatory effects of polyphenols have attracted much research attention, especially their potential therapeutic effects in hereditary iron overload though the underlying mechanisms are complex and not fully understood yet (Lesjak and Srai 2019; Milman 2020). Polyphenols are widely present in our diet, found in grains, legumes, nuts, vegetables, fruits, and beverages. The total flavonoid intakes in adults are between 200 and 400 mg/day (USA, 200 mg/d; South Korea, 318 mg/d; Europe, 370–437 mg/d) (Kim et al. 2016). Although there is no consensus on the definition of polyphenols, they can be broadly categorized into four main subgroups: flavonoids, phenolic acids, stilbenes, and lignans. Tea and coffee are flavonoid-rich beverages which contain high amounts of tannins. Drinking tea or coffee during meals substantially decreases non-heme iron absorption (Hallberg and Hulthen 2000). A single meal study showed that beverages containing 20–50 or 100–400 mg total polyphenols/serving reduced iron absorption from bread by 50–70% and 60–90%, respectively (Hurrell et al. 1999). Black tea (79–94%) has more iron suppressive effects than herbal teas like chamomile (47%), peppermint tea (84%), and cocoa (71%). Since milk is also a non-heme iron inhibitor, the addition of milk to tea or coffee has no beneficial effect on iron absorption (Hurrell et al. 1999). Hurrell et al. proposed an equation to determine the effects of polyphenol intake on iron bioavailability: relative iron absorption $= 2 \times (\text{mg polyphenol/serving})^{-0.5}$ (Hurrell et al. 1999). Black tea contains more tannins than green tea, herbal tea, cocoa, wine, or coffee. Tannins can be divided into condensed tannins (proanthocyanidins) and hydrolysable tannins (tannic acids). It is thought that condensed tannins are consumed in higher quantity than tannic acids (Delimont et al. 2017). Tannic acid and catechins in black tea and green tea can chelate ferric iron through three hydroxyl groups (galloyl) or two hydroxyl groups (catechol). A systemic review concluded that evidence from single meal studies supports the theory that tea and tannic acid consumption (hydrolysable and oligomeric catechin and epicatechin tannins) reduces iron bioavailability; however, evidence from epidemiologic and long-term human studies does not confirm the results of single meal studies (Delimont et al. 2017). The authors suggest that the effects of tea or tannin

consumption on iron bioavailability may greatly depend on the chemical structure of tannins (e.g., condensed tannins or tannic acids).

Polyphenols: Quercetin

Quercetin, one of the most extensively studied flavonoids, is a potent iron regulator. Quercetin can downregulate iron bioavailability through inhibiting iron absorption in the duodenum and iron efflux (intensively reviewed by Lesjak and Srai) (Lesjak and Srai 2019). Quercetin is found in diverse foods including vegetables (parsley, red cabbage, eggplants, onions, broccoli, garlic, tomatoes, spinach), fruits (blackberry, cranberry, banana, strawberry, figs, and apples), and tea. Daily quercetin intake in adults is approximately 15–18 mg/day; however, quercetin has a poor solubility and poor bioavailability (~2% absorption rate). Black and green teas provide good sources of dietary quercetin for regular tea drinkers. However, quercetin can reduce non-heme iron absorption by chelating metal ions through its 3-hydroxyl group, forming a stable complex with ferric iron. In addition, quercetin can regulate iron efflux through regulation of the hepcidin-FPN axis. In vitro and in vivo studies showed that quercetin upregulates expression of hepatic hepcidin but downregulates iron transporter FPN (Lesjak and Srai 2019). These results suggest that, although quercetin is poorly absorbed, it can transport to the bloodstream and control systemic iron homeostasis if consumed in high quantities. However, clinical trials are needed to confirm the iron regulatory effects of quercetin or flavonoids.

Proteins from Plants, Eggs, and Milk (Calcium)

Unlike the iron-solubilizing effects of low-molecular-weight peptides derived from muscle tissues, egg protein, soy protein, and milk protein decrease non-heme iron bioavailability. Legumes are iron-rich foods (6–7 mg/100 g), which also contain a better absorbed form of ferritin iron. However, legumes also have high content of phytates, polyphenols, and calcium. Soy protein decreases iron absorption in single meal test (Milman 2020). The non-heme iron inhibitory effects of soy protein are not fully understood, but inhibitors like phytate, polyphenols, calcium, or glycinin (11S) and conglycinin (7S) fractions of soy protein are likely to be involved. Fermented soy sauce or soy products (e.g., silken tofu, natto, tempeh, miso) improve iron absorption (WHO 2004). Although egg (exclusively in egg yolk) contains higher amount of iron (1.9–2.6 mg/100 g) than pork or chicken fillet (<0.5 mg/100 g), its iron bioavailability is low. Most of iron is present in egg yolks as insoluble ferric iron-phosvitin complex. The formation of insoluble iron sulfides during cooking may also decrease iron bioavailability of eggs. Cow milk, an extremely low-iron food (0.1–0.3 mg/100 ml), has high calcium (~100 mg/100 ml) and casein (~81% of milk protein) content. Both caseins and calcium are known to reduce iron bioavailability. Calcium reduces iron bioavailability of both heme and non-heme iron in a dose-dependent manner. 300–600 mg of calcium reduced iron absorption by 60% (WHO 2004). A typical calcium supplement contains >300 mg/tablet and therefore should be taken between meals to prevent interference with iron absorption after meals. Calcium may suppress iron exporter protein FPN in duodenum to prevent the release of dietary iron into the bloodstream (Lonnerdal 2010). However,

consumption of one serving of milk or yogurt (250 ml) to a plant-based diet has no effects on iron bioavailability (Milman 2020). This suggests that a moderate consumption of milk and dairy products has minimum effects on iron absorption. The addition of Vit C to meals enhances non-heme iron bioavailability of soy protein, eggs, and dairy products by preventing the formation of insoluble ferric iron complex or promoting the conversion of soluble ferrous iron.

Sugar

Sugar-sweetened beverages (e.g., glucose, fructose, high fructose corn syrup, aspartame) may decrease iron bioavailability (Christides and Sharp 2013), possibly through decreasing ceruloplasmin activity (Harder et al. 2020). Ceruloplasmin, a copper-containing enzyme with ferroxidase activity, plays an important role in iron transportation via the conversion of Fe^{2+} to Fe^{3+} (Harder et al. 2020).

Dietary Approaches for Iron Disorder

The World Health Organization (WHO) estimates that ID is the most common nutritional deficiency disorder and approximately 600–700 million people worldwide may have IDA (WHO 2004). Developing countries have high prevalence of absolute ID and true IDA, while functional ID and AI are more likely to occur in developed countries. The etiology and underlying mechanisms responsible for absolute ID and functional ID are distinctively different. Therefore, tailoring dietary approaches for subtypes of ID is needed.

Dietary Approaches for Absolute Iron Deficiency and Iron Deficiency Anemia

Body weight or body mass index is a good indicator of nutritional status. Absolute ID tends to occur in individuals who are underweight or living in poor countries. Poverty and malnutrition are intertwined. Malnutrition, vegetarian diets, and increased iron demand at some stages of life are major risk factors for absolute ID or true IDA. Specifically, insufficient intake of nutrients (e.g., protein, heme iron) to meet the demands of erythropoiesis is the main cause of true IDA. Although vegetarians or vegans (lacto-ovo-vegetarian) usually have high intake of dietary iron, they are more likely to have lower body iron stores (indicated by serum ferritin) compared with non-vegetarians. This is likely due to the low bioavailability of non-heme iron in plant-based foods. Patients with absolute ID or true IDA normally have good dietary responses to iron-containing agents (e.g., iron-rich foods, iron-fortified foods, iron supplements). The dietary approaches for true IDA patients are to increase consumption of iron-rich foods and increase foods that enhance iron absorption (iron enhancers) while reducing intake of iron inhibitory foods (see Table 3). Tips for meal planning are (1) eating iron-rich food (e.g., red meat, liver) or taking iron-fortified cereals or bars; (2) eating Vit C-rich fruits/juices or taking Vit C supplements during meal time; (3) choosing β-carotene-rich carbohydrate, fruits, or vegetables (e.g., sweet potato, potato, tarot, pumpkins, mango, papaya, carrot) or

low phytate breads (e.g., sourdough bread instead of whole grain breads), noodles, and white rice; (4) reducing intake of high phytate foods [e.g., whole grains (brown rice, wheat bran), legumes, green leafy vegetables, or nuts] and instead choosing low phytate vegetables (roots, tubers, e.g., carrot, radish, turnip, parsnip), processed grains (e.g., flour- or iron-fortified cereals), or processed/fermented legumes (soy milk, tofu, miso instead of soybean); and (5) iron suppressing beverages (e.g., tea, coffee, wine, cocoa, or milk) should be taken in moderate quantity and between meals.

Oral iron supplementations have moderate protective effects against anemia [RR: 0.34 (0.20 to 0.57)] and ID [RR 0.61 (0.47 to 0.77)] in menstruating women and adolescents (WHO 2006). Hence, the WHO recommends that, for menstruating women/adolescents (WHO 2006) and children (age 5–12 years) (WHO 2016a) living in regions with high anemia prevalence (≥40%), a daily dose of 30–60 mg elemental iron [ferrous sulfate heptahydrate (150–300 mg/day), ferrous fumarate (90–180 mg/day), ferrous gluconate (250–500 mg/day)] should be taken for 3 months. For anemic pregnant women, a daily dose of 120 mg elemental iron is recommended until her Hb levels return to normal (WHO 2016b). After that, a standard antenatal dose (60 mg iron +400 μg folate) should follow. A daily dose of 30 mg elemental iron taken consecutively for 3 months is recommended for children and infants (24–59 months of age) where anemia prevalence is high (WHO 2016a). However, oral iron supplementation needs to take into account of the use of iron-containing agents (e.g., ferrous or ferric iron, multivitamins, iron-fortified foods) or the presence of infection (e.g., malaria, parasite). Use of polyphenol supplements (e.g., quercetin, resveratrol) should be treated with caution when body iron levels are low (Lesjak and Srai 2019; Milman 2020).

Dietary Approaches for Functional Iron Deficiency and Anemia of Inflammation

Obesity is one of the strongest risk factors for functional ID. A recent meta-analysis showed that, compared to normal-weight individuals, obese adults and children have a 1.3-fold (OR 1.3, 95% CI 1.01–1.68) (Zhao et al. 2015) and 2.1-fold (OR 2.1, 95% CI 1.4–3.2) (Malden et al. 2021) increased risk for functional ID. Coexistence of low serum iron and mild tissue iron overload may occur in patients with obesity or chronic diseases. Iron supplementation should be avoided if iron overload is present. Currently, there are no consensus statements on the dietary recommendations for treating patients with functional ID and AI. Nonetheless, patients should follow healthy eating guidelines and maintain a healthy body weight. Diet-induced weight loss may help re-establish iron homoeostasis in people who are overweight or obese (Teng et al. 2020). An average of 5 kilogram (kg) [weighted mean differences (WMD) = −4.60 kg, 95% CI: −6.74, −2.47 kg] weight loss significantly increased transferrin saturation (Tsat) [WMD = 1.68%, 1.68%, 0.97–2.39%, $I^2 = 44\%$] (Teng et al. 2020).

Unlike absolute ID, nutrient deficiency is not the primary cause of functional ID. Obese people usually consume more meat (especially red meat), which contains highly absorbed heme iron or MFP than under- or normal-weight individuals. However, they also have lower intake of anti-inflammatory foods such as omega-3

polyunsaturated fatty acids (n-3 PUFA) and polyphenol-containing foods. Since inflammation, and not micronutrient deficiency, is the underlying cause of functional ID and AI, consumption of anti-inflammatory foods may help reduce inflammation and restore iron homeostasis. An anti-inflammatory diet, based on the dietary inflammatory index originally proposed by Hébert JR, refers to a group of foods with anti-oxidative stress and anti-inflammation modulating effects (Hebert et al. 2019). An anti-inflammatory diet encourages the consumption of whole grains, polyphenol-rich legumes, polyphenol-vitamin C-ß-carotene-rich fruits, polyphenol-ß-carotene-vitamin D-rich vegetables, spices (garlic, ginger, turmeric), polyphenol-rich tea (black and green), lean meat, and foods containing omega-3 PUFA (fatty fish, nuts, seeds). By contrast, intake of refined carbohydrates, sugar, red meat, processed foods, trans fat, saturated fat, deep-fried food, and alcohol should be limited. Foods that trigger inflammation may also promote hepcidin production. Human studies have shown that high intake frequency of meats and processed meats, deep-fried food, and animal fat, but low intake of steamed/boiled/raw foods and dairy products are associated with elevated serum hepcidin and ferritin in obese adults (Cempaka et al. 2019). By contrast, increased intake of anti-hepcidin foods may help. A one-time high dose of vitamin D supplementation (250 000 IU) significantly decreased serum hepcidin in healthy adults (Smith et al. 2017). Decreased hepcidin levels may help release tissue trapping iron and restore iron homeostasis in obesity.

Overall, evidence on how anti-inflammatory foods regulate hepcidin synthesis and iron homeostasis is scarce and more human studies are needed. Nonetheless, dietary strategies to maintain healthy body weight and lower the levels of inflammation, and thus hepcidin, may have therapeutic value for functional ID and AI. Replacing red meat with n-3 PUFAs and Vit D-rich oily fish (eel, mackerel, salmon, sea bass, milk fish), eating Vit C and β-carotene-rich fruits during meals, and drinking polyphenol-rich black and green tea between meals may prevent the progression of functional ID to AI. Although polyphenols exhibit potent anti-inflammatory effects, flavonoids (e.g., quercetin) are strong iron chelators and promote hepcidin synthesis (Lesjak and Srai 2019; Milman 2020). Hence, patients should have moderate consumption of flavonoid-containing foods and not take high doses of polyphenol supplements [e.g., quercetin, genistein, resveratrol, turmeric (3.2 g/day)] to prevent IDA.

Dietary Approaches for Hereditary Iron Disorders

The commonly seen hereditary iron disorder diseases include hereditary hemochromatosis (HH) in Caucasians, sickle cell anemia in black people, and thalassemia in Asians or South Europeans. Anemia may coexist with iron overload due to ineffective erythropoiesis, regular blood transfusion, or excessive iron absorption. Mutation of the HFE genes (primary or secondary) causes hepcidin deficiency leading to excessive iron absorption. Anemia or ineffective erythropoiesis also suppresses hepcidin production. Hence, patients with hereditary iron disorders usually absorb more dietary iron, predisposing them to iron overload. Phlebotomy is the principal treatment method for removing excess body iron and preventing iron overload.

Iron overload is caused by an imbalance of iron absorption and body iron requirement. These patients should avoid consuming iron-rich foods (red meat, liver, blood), iron-fortified cereals, snacks, or sports drinks or taking iron supplements (multivitamins and minerals). Increased intake of nutrients that aid erythropoiesis (folate, Vit B6/12) or protect RBC membrane integrity (antioxidants) is encouraged. Calories and proteins should be given in sufficient quantities for children and pregnant women. For adults, maintaining a healthy body weight, or energy restriction, may prevent iron overload through enhancing hepcidin secretion.

In summary, patients with hereditary iron disorders may adapt a low-iron diet and increase foods that are known to inhibit iron bioavailability (see Table 3). A well-balanced diet with foods rich in plant proteins, folate (400–600 mg/d), Vit B6 and 12, copper, zinc, polyphenols, antioxidants (ß-carotene, Vit E) and anti-inflammatory foods (n-3 PUFA, Vit D, polyphenols), but low in heme iron, processed and deep-fry foods, and alcohol is recommended. Although polyphenol exerts strong anti-oxidative and iron-chelating ability, the therapeutic potential of polyphenol supplements for preventing iron overload in humans is limited and inconclusive (Lesjak and Srai 2019; Milman 2020).

Diagnosis of Iron Disorder Diseases

Absolute Iron Deficiency and Iron Deficiency Anemia

Absolute ID and IDA are the most well-characterized iron disorder disease.

- **Hb.** According to the WHO, normal Hb values in males and females are >130 g/l and >110–120 g/l, respectively. Children aged 6–59 months, 5–11 years (yrs), and 12–14 yrs. should have Hb levels exceeding 110, 115, and 120 g/l, respectively. Reduction of Hb levels below these values corresponds to IDA of different severity (mild, moderate, severe) (WHO 2011). Although blood Hb levels provide substantial contribution to diagnosis of IDA, other markers of iron metabolism provide additional information for differential diagnosis of IDA.
- **Ferritin.** Ferritin has been used as a marker of iron status with a serum concentration of <15 μg/L indicating absolute ID (Daru et al. 2017). At the same time, there are concerns regarding this cut-off (Daru et al. 2017) as well as limitations of using ferritin for body iron assessment.
- **Transferrin-related biomarkers.** Tsat is a sensitive marker of low iron status, with values of <15–20% corresponding to absolute ID irrespective of inflammation (Cacoub et al. 2019). Corresponding to reduced Tsat, total iron-binding capacity (TIBC) is elevated in patients with IDA. Another transferrin-related biomarker of iron status is the soluble transferrin receptor (sTfR). Both sTfR and sTfR/log ferritin index are considered as positive predictors of IDA.
- **Hepcidin.** Although not used for routine laboratory diagnosis of IDA, hepcidin plays a key role in modulation of iron metabolism in a variety of (patho)physiological conditions. Specifically, both pro-hepcidin and hepcidin are reduced in patients with absolute ID and IDA, with the latter being positively associated with

Hb levels, mean corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) values, as well as serum iron, ferritin, and TS.

- **Other biomarkers.** Zinc protoporphyrin (ZnPP) has also been used for ID screening.

Functional Iron Deficiency and Anemia of Inflammation

Functional ID is characterized by normal iron stores but insufficient availability of iron for processes including erythropoiesis. The most common states characterized by functional ID are anemia of chronic kidney disease, anemia of chronic disease (ACD), or AI. Anemia of chronic kidney disease is caused by insufficient production of EPO, whereas in AI/ACD, hepcidin appears to be the key player resulting in iron sequestration (Weiss et al. 2019). AI/ACD is associated with a wide spectrum of pathologies characterized by systemic inflammation including infectious and inflammatory/autoimmune diseases, cancer, chronic heart failure, chronic pulmonary disease, anemia of critical illness, and obesity (Weiss et al. 2019). AI/ACD is also frequently observed in elderly patients due to the presence of multiple age-related pathologies.

Pathogenetic aspects of AI are discussed in a number of excellent reviews by Ganz, Nemeth, and Weiss (Weiss et al. 2019; Nemeth and Ganz 2014). Therefore, only a brief description of AI/ACD pathogenesis will be provided. Systemic inflammation is associated with overproduction of proinflammatory cytokines, namely, IL-6, IL-1β, and TNF-α, that upregulate hepcidin expression and secretion by the liver and other tissues. These factors also downregulate EPO production. Increased circulating hepcidin levels result in iron sequestration in the reticuloendothelial system through inhibition of FPN. The latter is also responsible for hepcidin-induced decrease in intestinal iron absorption. In addition to reduced EPO production, systemic inflammation may also result in erythroid progenitor cell damage, further aggravating erythropoiesis. Taken together, the cascade of inflammatory response and hepcidin upregulation result in iron sequestration in macrophages, thus reducing its availability for erythropoiesis (Tables 4 and 5).

Diagnosis of AI/ACD, as well as its differential diagnosis from IDA, is essential for designating the appropriate treatment strategy. On the one hand, low Hb levels are characteristic for both IDA and ACD, although in the latter case a severe decrease of Hb levels is unlikely. Due to the presence of chronic inflammation, the patterns of iron status markers in functional ID and AI/ACD are quite different from those observed in IDA (Table 6).

- **Ferritin.** Given the nature of ferritin as an acute phase protein, inflammation has a significant impact on its circulating levels, thus limiting its diagnostic value (Dignass et al. 2018). A meta-analysis demonstrated that inflammation assessed by circulating C-reactive protein and alpha 1 acid glycoprotein levels increased ferritin concentration by ~30%, thus resulting in 14% underestimation of anemia. Therefore, adjustment of ferritin levels for inflammatory markers is essential for improvement of its informativity in diagnosis of AI/ACD (Namaste et al. 2017).
- **Transferrin saturation.** A recent systematic review also indicated the usefulness of Tsat assessment for evaluation of AI/ACD. Tsat was found to be lower in the

Table 4 Dietary approaches for functional iron deficiency and anemia of inflammation

Nutrient	Increase or consider	Decrease, avoid, or treat with caution
Protein	n-3-PUFA or Vit D foods -Oily fish (eel, mackerel, salmon, sea bass, milk fish) -Egg, dairy products	Red meat, livestock, goose, duck
	-Lean meat -Plant-based (soy, tofu, legumes)	Organs, blood products
Carbohydrate	Unprocessed carbohydrate	Refined carbohydrate (e.g., white bread, rice)
	(whole grains, brown rice, oatmeal, quinoa/djulis, sweet potato)	
Vegetables	Polyphenol- or ß-carotene-rich vegetables	
	(parsley, red cabbage, eggplants, onions, broccoli, garlic, tomatoes, spinach)	
Fruits	Vit C, ß-carotene, quercetin-rich fruits (blackberry, cranberry, banana, strawberry, figs, mango, apples)	
Oils	n-3 PUFA and Vit E and MUFA (chia seed, flax seed, Thai basil seeds, walnut, nuts), avocados	Saturated fat (animal fat, palm oils, coconut oils)
Beverages	Tea, coffee	Alcohol, sugar-sweetened beverages
Supplements	Vit C, Vit D, n-3 PUFAs	Iron-containing supplements or iron-fortified foods Polyphenol (e.g., quercetin, genistein, resveratrol, turmeric)

Table 5 Dietary approaches for hereditary iron disorders

Nutrients	Increase or consider	Decrease, avoid, or modify
Protein	Plant-based (soy protein, tofu, legumes)	Red meat, livestock, goose, duck,
	Fatty fish, shell fish, dairy, eggs, lean meat	Organs, blood products
Carbohydrate	Unprocessed carbohydrate (whole grains, legumes, oatmeal, quinoa, djulis)	Refined carbohydrate (e.g., white bread); iron-fortified flour/cereals
Vegetables	Folate- and polyphenol-rich vegetables	
	(parsley, red cabbage, eggplants, onions, broccoli, garlic, tomatoes, spinach)	
Fruits	Quercetin- and folate-rich fruits (blackberry, cranberry, banana, strawberry, figs, and apples)	Eat Vit C-rich fruits between meal time to prevent non-heme iron absorption
Oils	n-3 PUFA and Vit E (chia seed, flax seed, Thai basil seeds, walnut, nuts), avocados	Saturated fat (animal fat, palm oil, coconut oils)
Beverages	Tea, coffee (during meal)	Alcohol, sugar-sweetened beverages, Vit C-fortified drinks
Supplements	Multivitamins and minerals, Vit E, polyphenol (e.g., quercetin, curcumin)	Iron-containing supplements or iron-fortified foods Vit C supplements

Table 6 Hematological and biochemical markers in patients with iron deficiency anemia, anemia of chronic disease, or both pathologies

	IDA	ACD	IDA + ACD
Hb	Low	Low	Low
Serum iron	Low	Low	Low
Ferritin	Low	High	Normal-to-high
Transferrin	High	Low-to-normal	Low-to-normal
Transferrin saturation	Low	Low	Low
sTfR	High	Normal	Normal-to-high
sTfR/log ferritin	High (>2)	Low (<1)	High (>2)
CRP[a]	Low	High	High
Hepcidin	Low	High	Normal-to-high
Erythropoietin	High	Normal	High
GDF-15	Normal	Normal-to-high	High

[a]Or other markers of inflammation including proinflammatory cytokines

cases of AI/ACD, while serum ferritin was characterized by an increase, in contrast to IDA, when both biomarkers were low (Cacoub et al. 2019).

- **Soluble transferrin receptor.** Being a valuable marker of systemic iron status, sTfR is also used as a tool for discrimination of IDA from AI/ACD. However, inflammatory response was also shown to affect sTfR levels, and adjustment of inflammatory markers could provide additional value (Rohner et al. 2017). Evaluation of sTfR/ferritin index revealed significantly higher values in patients with IDA or ACD + IDA as compared to ACD alone, yielding more than a two-fold increase (41–92%) in IDA diagnosis for the use of a panel of ferritin, sTfR, and sTfR index as compared to ferritin alone. The use of sTfR-ferritin index was shown to improve the diagnosis rate of ID in inflammatory bowel disease patients by 36% as compared to ferritin only (Abitbol et al. 2015).
- **Hepcidin.** Due to the role of hepcidin as a key player in development of AI, studies have investigated its predictive value for diagnosis of AI. Specifically, circulating hepcidin levels were found to be increased in patients with AI, being inversely correlated with sTfR/log ferritin, serum iron, Hb, and TIBC (Khalaf et al. 2019) and positively associated with IL-6, ferritin, and creatinine concentrations (Suega and Widiana 2019). A previous study demonstrated that hepcidin concentrations are characterized by nearly two-fold higher levels in AI as compared to controls, whereas increased circulating hepcidin levels could be considered as a sensitive and specific marker of AI (Vyas et al. 2018). Correspondingly, a recent meta-analysis revealed that the summary receiver operating characteristic curve value for the use of hepcidin levels was 0.91 (Han and Wang 2021). However, the sensitivity of hepcidin may be affected by the rate of inflammation.
- **Erythropoietin.** Being associated with hepcidin, inflammation, and iron status, erythropoietin (EPO) levels are characterized by specific patterns in ACD. Circulating EPO levels in patients with ACD were found to be nearly 50% lower

than those observed in IDA (Gowanlock et al. 2016). At the same time, anemia was shown to affect the association between inflammation and EPO response.

- **Growth differentiation factor 15 (GDF).** GDF is a cytokine induced in response to a variety of stress signals including inflammation, oxidative stress, and mitochondrial dysfunction (Moon et al. 2020). Due to its involvement in multiple metabolic pathways, GDF15 is considered as a potential biomarker of cardiovascular diseases, diabetes, cancer, etc. (Arkoumani et al. 2020). Given the role of GDF15 in inflammatory responses and hepcidin signaling, it is considered as a potential marker of iron status. It has been demonstrated that GDF15 levels were significantly higher in patients with ACD and especially ACD/IDA as compared to IDA, being associated with sTfR values, but not circulating hepcidin. However, no significant difference between control and IDA groups was observed.
- **Metabolomic markers.** In addition to routinely used markers, certain studies have evaluated the diagnostic potential of various metabolomic panels. Specifically, based on the findings of metabolomic analysis in patients with IDA, AI, and IDA + AI, a panel of eight biomarkers including apolipoprotein A4, transferrin, TfR1, ceruloplasmin, haptoglobin, lactoferrin, hemopexin, and matrix metalloproteinase-8 was developed. Compared to the routinely used transferrin the panel increased the diagnostic accuracy in IDA from 94% to 100%, in AI 50% to 72%, and in IDA + AI group from 66% to 96% (Domanski et al. 2012). A recent study demonstrated the potential usefulness of ZnPP assessment for the evaluation of iron status under inflammatory conditions (Leventi et al. 2021).

Hereditary Iron Disorder Diseases

Hemochromatosis

HFE is a hereditary iron overload disorder caused by mutations of genes involved in the regulation of iron metabolism. Specifically, according to the genes involved in the pathogenesis of HH, the disease is classified into four types: I, HFE; IIa, HJV; IIb, HAMP; and III, TFR2, all being autosomal recessive, as well as IV – SLC40A1 (FPN disease), which is autosomal dominant (Kawabata 2018). Of these four HH types only the first is considered HFE-hemochromatosis, being correspondingly graded into subtypes according to the variants: (a) HFE Cys282Tyr homozygosity, (b) HFE Cys282Tyr/His63Asp compound heterozygosity, and (c) other minor genotypes including Ser65Cys (Gerhard et al. 2018). Analysis of HH genetic types demonstrated more severe iron overload in patients with non-HFE types with mutations of HJV, HAMP, and TFR2 genes (Sandhu et al. 2018), in agreement with clinical and laboratory patterns (Table 7).

Diagnostic investigation of HH starts from the evaluation of clinical features in symptomatic patients, genetic anamnesis, or estimated iron overload. Quantification of hepatic iron accumulation is the key method for diagnosis of HH. Magnetic resonance imaging is considered as the standard technique for assessment of hepatic iron accumulation, being more sensitive and specific than ultrasound or computerized tomography (Golfeyz et al. 2018). Cases at high risk of hereditary HH are subjected to evaluation of iron markers.

Table 7 Genetic and clinical characteristics of hereditary hemochromatosis types

Type	I	IIA	IIB	III	IV
Gene	*HFE*	*HJV*	*HAMP*	*TFR2*	*SLC40A1*
Protein	HFE	Hemojuvelin	Hepcidin	Transferrin receptor 2	FPN
Dysfunction	Hepcidin	Hepcidin	Hepcidin	Iron transport	Iron export
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal recessive	Autosomal dominant
Hepcidin	Low	Low	Absent	Low	High
Age of onset	Adult	Juvenile	Juvenile	Juvenile/ adult	Adult
Clinical features	Classic hemochromatosis	High severity. hypogonadism, and cardiomyopathy		Higher severity than type I	Anemia. reduced end-organ damage

- **Transferrin saturation.** Tsat is considered as the first investigative laboratory tool. Patients with Tsat >45% are considered at risk of HH and should be subjected to HFE gene testing (Makker et al. 2015). Moreover, in a follow-up study, patients with high Tsat (>50%) were characterized by a more severe joint symptoms, as well as reduced athletic capacity, work ability, and libido.
- **Ferritin.** More than 50% of C282Y homozygous HH patients were characterized by ferritin levels exceeding 1000 μg/L (Gasser et al. 2014). Ferritin levels were found to correlate significantly with hepatic T2*MRI values of iron content. Correspondingly, serum ferritin levels >1000 μg/l in HFE patients are associated with significantly higher mortality risk, even in those undergoing treatment. At the same time, certain inconsistencies in different diagnostic approaches exist. Particularly, a recent case report demonstrated inconsistency between high serum ferritin levels and liver iron content as assessed by T2*MRI that did not reveal increased iron accumulation (Al-Tikrity and Yassin 2020).
- **Hepcidin.** Although not a standard marker, serum hepcidin levels may be used for differential diagnosis of iron overload, being significantly reduced in HH patients, whereas FPN disease and secondary iron overload patients were characterized by serum hepcidin levels correlated to ferritin concentrations (Kaneko et al. 2010).
- **Molecular diagnostics.** Given the genetic cause of HH, the first line of molecular diagnostic is testing of HFE gene for p.Cys282Tyr and p.His63Asp variants corresponding to type 1 HFE (C282Y/C282Y, C282Y/H63D). In the case of distinct genotypes other causes of HFE are to be considered (Fig. 2). Genotyping for TFR2, SLC40A1, HAMP, and HJV variants will provide essential information for the diagnosis of type 3, 4, 2A, and 2B hemochromatosis, respectively. Early onset and endocrine manifestations and cardiomyopathy may also contribute to the diagnosis of the latter two subtypes.

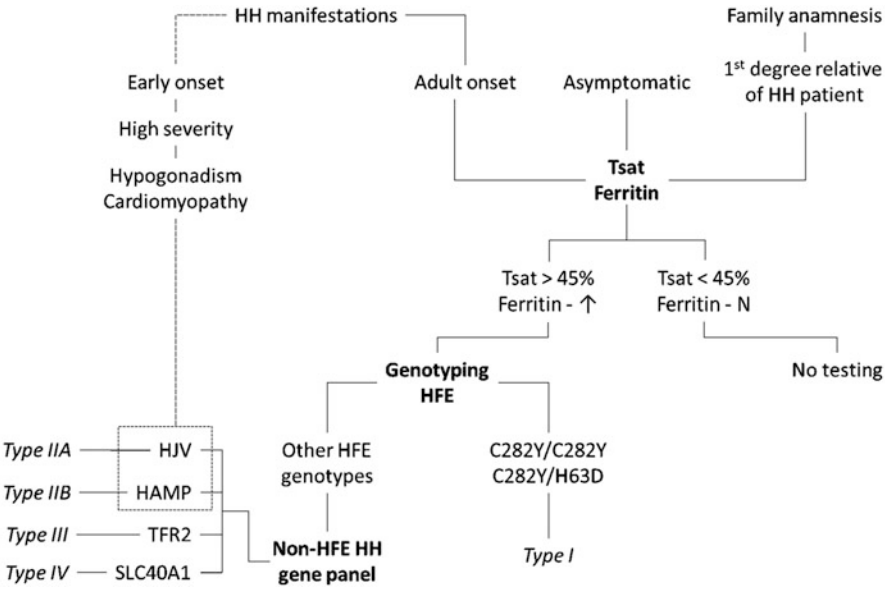

Fig. 2 Diagnostic strategy in patients at high risk of hereditary hemochromatosis

β-Thalassemia

β-Thalassemia is an autosomal recessive disorder caused by alterations in Hb β-globin chain synthesis. Due to genotype variations associated with absolute lack (β^0) or reduced (β^+) β chain synthesis, β-thalassemia may be characterized by carrier state/β-thalassemia minor, β-thalassemia intermedia (TI), and β-thalassemia major (Table 8). Diagnosis of β-thalassemia is based on clinical and hematological features and evaluation of iron status with subsequent evaluation of Hb and estimation of the underlying genetic background.

- **RBC indices.** Due to complex alterations of erythropoiesis, β-thalassemia is characterized by reduced Hb levels, low MCH and MCV values, as well as multiple morphological alterations of RBCs including microcytosis, anisocytosis, poikilocytosis, and hypochromia. MCV and MCH are considered as the valuable tool for β-thalassemia screening.
- **Ferritin.** Thalassemia is characterized by secondary iron overload, thus being associated with high ferritin levels that are known to be significantly associated with hepatic iron accumulation as assessed by liver T2*MRI. Moreover, in a 10-year cohort follow-up study, high serum ferritin levels in β-thalassemia intermedia were associated with a significantly increased morbidity including osteoporosis, extramedullary hematopoiesis, liver diseases, etc. (Musallam et al. 2012). However, no significant difference in ferritin levels was observed for patients with β^0- and β^+-associated thalassemia.
- **Hepcidin.** Being a key regulator of systemic iron metabolism, hepcidin levels are also affected in β-thalassemia (Nemeth 2013). Specifically, in β-thalassemia

Table 8 Characteristics of β-thalassemia with different genotypes and disease severity

	Major	Intermedia	Minor/trait
Common genotypes	β^0/β^0	β^+/β^0 β^+/β^+	β/β^0 β/β^+
Hb, g/l	Hb < 7 g/l	7 < Hb < 10 g/l	Borderline low-normal
MCV, fl	50 < MCV < 70 fl	50 < MCV < 80 fl	Borderline low-normal
MCH, pg	12 < MCH < 20 pg	16 < MCH < 24 pg	Borderline low-normal
HbF, %	HbF > 90%	30 > HbF > 90%	HbF < 30%
Extramedullary hemopoiesis	Yes	Possible	None
Iron overload	Yes	Yes	None
Transfusion	Transfusion-dependent	Non-transfusion-dependent	Non-transfusion-dependent

intermedia hepcidin deficiency correlates with EPO, sTfR, and other markers of erythropoiesis. However, in β-thalassemia major, hepcidin levels were increased in response to hemotransfusions.

- **Hb spectrum.** Due to the role of Hbopathy as a pathogenetic consequence of β-thalassemia, evaluation of thalassemia types is performed through assessment of the Hb spectrum, particularly the percentage of HbF and HbA2 out of total Hb levels (Brancaleoni et al. 2016). Briefly, the highest HbF percentage (up to 95%) is observed in β^0 homozygotes (β-thalassemia major).
- **Genetic analysis.** Genetic analysis of HBB gene mutations using PCR-based procedures, scanning, or sequence analysis is used as the final step of molecular diagnosis. Currently, 279 variants associated with β-thalassemia are estimated, according to the HbVar database (Sabath 2017).

Conclusion

Mechanisms underlying iron restriction and iron overload are different from absolute ID. Understanding how hepcidin integrates multiple signals (e.g., diet, gene, body iron, inflammation) to control iron bioavailability may help improve diagnostic accuracy and translate into making effective dietary approaches for patients with different types of iron disorder.

Applications to Prognosis

In this chapter we review the relationship between hepcidin and iron disorder diseases and discuss dietary and nutritional approaches for the management of iron disorders. Specifically, this chapter focuses on diet plans for absolute ID, functional ID and AI, and hereditary iron overload. Preliminary studies suggest that

phytonutrients such as flavonoids may have therapeutic potential in hereditary iron overload. Flavonoids like quercetin in fruits, vegetables and spices, or tannins in tea and coffee are potent iron inhibitors, which may chelate non-heme iron or enhance hepcidin synthesis. Weight loss and anti-inflammatory diets may help restore iron homeostasis in patients with functional ID or AI. However, it is important to note that most available evidence for iron bioavailability comes from cell culture, animal studies, or single meal tests. The effectiveness of nutritional therapy for functional ID, AI, and iron overload remains to be confirmed in well-designed clinical trials.

Mini-Dictionary of Terms

- **MFP factors.** *Iron-binding low-molecular-weight peptides released by digestion which bind iron in a soluble form in the stomach. This prevents chelation of non-heme iron by dietary inhibitors (*e.g.*, phytic acid and polyphenols) and enhances non-heme iron absorption from other foods eaten in the same meal.*

  Absolute iron deficiency. *It occurs when dietary iron intake does not meet the body's iron requirements. Individuals who are suffering from malnutrition, vegetarian, living in poverty, or at special life stages have higher risk.*
- ***Functional iron deficiency.*** *A new type of iron disorder induced by inflammation that may lead to anemia of inflammation. Patients usually have low serum iron levels despite adequate body iron stores. There is not yet a consensus on the diagnosis but elevated serum hepcidin, low serum iron, and high body iron store (ferritin levels are normal or elevated) are observed. Impairment in iron utilization, not insufficient iron intake, is the main cause. Patients tend to have decreased iron absorption rate due to high hepcidin levels.*
- ***Iron overload.*** *Accumulation of iron in the body. Patients with primary hemochromatosis have a two- to three-fold increase in dietary iron absorption due to hepcidin deficiency. Gradually, patient may develop severe liver iron overload. Other potential causes include hepatitis (viral, alcohol, high fat or high fructose diet), obesity-related comorbidities, excessive intake of iron-fortified foods or supplements, as well as regular blood transfusion.*
- ***Anti-inflammatory diet.*** *A type of balanced diet focused on nutrients with anti-inflammatory and anti-oxidative functions such as polyphenols, ß-carotene, vitamins A/C/D/E, and omega-3 PUFAs. This diet avoids foods that promote inflammation (*e.g.*, refined carbohydrate, sugar, red meat, processed foods, trans and saturated fat, and alcohol). Consumption of vegetables, fruits, whole grains, legumes, fatty fish and sea foods, spices, nuts and seeds, tea, and coffee is also encouraged.*

Key Facts of Anemia of Inflammation

- *Frequently observed in obesity-related comorbidities or chronic diseases.*
- *Inadequate iron intake is not the cause.*

- *Inflammation triggers overproduction of hepcidin, resulting in hepcidin-mediated proteolytic degradation of iron exporter ferroportin.*
- *Alteration in hepcidin-ferroportin axis causes insufficient mobilization of iron from iron stores to the circulation.*
- *Coexistence of high iron stores and mild to moderate anemia (Hb rarely < 8 g/dL) may occur.*
- *Maintaining a healthy body weight and adapting anti-inflammatory diet may help restore iron homeostasis.*
- *Iron supplementation is not recommended if iron overload is present. Treatment should focus on the underlying disease and anemia may improve once inflammation is controlled.*

Summary Points

- Iron deficiency remains the most common nutritional disorder worldwide.
- Tight regulation of iron balance is critical to prevent iron overload and iron deficiency.
- Hepcidin, a master iron regulator, controls iron balance through proteolytic degradation of ferroportin, the only known mammalian iron exporter.
- Insufficient hepcidin is central to hereditary iron overload.
- Uncontrolled inflammation promotes hepcidin overproduction and triggers functional iron deficiency and anemia of inflammation.
- The etiology of iron restriction and iron overload is distinct from absolute iron deficiency.
- Advances in the understanding of hepcidin and its interactions with diet may help translate into making effective dietary approaches for patients with different types of iron disorder.

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Glycolate as a Biological Marker of B Vitamins

12

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Abstract

Recent research results have revealed that the nutritional status of B vitamins and glycolate (glycolic acid, GA) metabolism are closely related. GA is the smallest alpha-hydroxy acid and is found in various foods. In this text, we reviewed the properties, digestion, absorption, metabolism, and excretion of GA. We also summarized the relationship between B vitamins, especially vitamins B2 and B6, and GA. Vitamins B2 and B6 act as cofactors for enzymes important in GA metabolism, and their deficiency is linked to changes in GA levels. Finally, the potential of GA as a deficiency marker for vitamins B2 and B6 was presented.

Keywords

Glycolate · B vitamins · Vitamin B2 · Vitamin B6 · Glycolate oxidase · Alanine/glyoxylate aminotransferase 1 · Glyoxylate · Flavin adenine dinucleotide · Flavin mononucleotide · Pyridoxal 5′-phosphate

Abbreviations

AGT1	Alanine/glyoxylate aminotransferase (protein)
Agxt1	Alanine/glyoxylate aminotransferase 1 (gene)
Agxt2	Alanine/glyoxylate aminotransferase 2 (gene)
ATP	Adenosine triphosphate
BW	Body weight
EGRAC	Erythrocyte glutathione reductase activating factor
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GA	Glycolate (glycolic acid)
GO	Glycolate oxidase
GRHPR	Glyoxylate reductase/hydroxypyruvate reductase
Hao1	Hydroxyacid oxidase-1
HOGA1	4-Hydroxy-2-oxoglutarate aldolase
Hyp	Hydroxyproline
LDH	Lactate dehydrogenase
MCT	Monocarboxylic acid transporter
mRNA	Messenger ribonucleic acid
PH	Primary hyperoxaluria
Pl	Pyridoxal
PLP	Pyridoxal 5′-phosphate
Pm	Pyridoxamine
PMP	Pyridoxamine 5′-phosphate
Pn	Pyridoxine
PPP	Pentose phosphate pathway
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism

USA	United States of America
VB2	Vitamin B2
VB6	Vitamin B6

Introduction

Vitamins are essential nutrients, and their inadequate intake can cause various abnormalities in living organisms. To prevent insufficient intake of vitamins, it is necessary to know the vitamin nutritional status of an organism. Many vitamin deficiency markers have been identified so far. Recently, advances in measurement instruments, such as mass spectrometers, have made it easier to analyze nontarget compounds and to perform comprehensive metabolite analysis. In this process, new vitamin B deficiency markers have been discovered. In this review, we summarize the properties of GA as a candidate marker for vitamin B2 (VB2) or B6 (VB6) deficiency.

Glycolate

Glycolate (GA) is the smallest α-hydroxy acid ($C_2H_4O_3$, HO-CH_2-CO_2H). It is odorless, colorless, and water-soluble. Vegetables, fruits, and beverages contain significant amounts of GA, and meat and milk contain much smaller amounts (Harris and Richardson 1980). The amount of GA present in 27 vegetable samples ranged from 0.5 to 5.6 mg per 100 g fresh wet weight. In 15 fruit samples, its content ranged from 0.45 to 7.4 mg per 100 g flesh weight. GA in hot beverages from plant sources ranged from 2.6 to 13.6 mg/228 g serving, and GA was found in root beer (3.7 mg/342 g serving). The GA content of meats (0.1–0.5 mg/100 g raw meat) was considerably less than that of vegetables and fruits. In humans, reference ranges (mean ± 2SD) of plasma GA (≤14 nmol/mL) and urine GA/creatinine ratio (≤50) were reported in 67 healthy adults at Mayo Clinic (McGregor et al. 2020). GA is also produced from the metabolism of hydroxyproline (Hyp), the final product of animal protein, and the collagen turnover (Wu et al. 2016). GA is used as an excipient in cosmetics and some pharmaceuticals. Available nonclinical data have shown no serious safety concerns with GA, such as genotoxicity or carcinogenicity, when used typically at low concentrations. Although low in toxicity per se, glyoxylates and oxalates, which are metabolites of GA, are toxic in mammals. Because GA occurs naturally in the diet and contributes to the formation and excretion of oxalate in the urine, limiting the intake of GA may be beneficial in treating patients with oxalate stones (Harris and Richardson 1980). The details of the GA metabolism are discussed in a separate section.

Intestinal Absorption of GA

GA is readily absorbed from the upper gastrointestinal tract, mainly the jejunal region. In rats, the absorption process of GA had a Km of 6.25 mM and a Vmax rate of 5.56 μmole/30 min/g wet weight (Talwar et al. 1984). In male Wistar rats,

orally administered sodium GA (39 to 776 mg/kg body weight) was excreted in the urine in a dose-dependent manner. In another study, after oral administration of [1-^{14}C] GA to rats at dosage levels from 0.51 to 10.20 mmole per kg of body weight, less than 3% of the administered radioactivity was excreted in the feces in 48 h (Harris and Richardson 1980). There were no differences in fecal excretion of GA between fasted and nonfasted rats, and changes in the content of the stomach and intestine because of fasting and dietary minerals do not influence the absorption of GA. This result indicates that GA is readily absorbed in large amounts from the gastrointestinal tract.

Cellular Uptake of GA

Uptake of GA by the human hepatocarcinoma cell line HepG2 was rapid, and the intracellular GA concentration reached equilibrium 5 min after the addition of 1 mM GA in the medium (Baker et al. 2004). The intracellular GA concentration equilibrated at 70% of the concentration in the medium. This rapid equilibration across the cell membrane is consistent with the transport properties of the proton-coupled monocarboxylic acid transporter (MCT) (Jackson and Halestrap 1996; Wang et al. 1996). Indeed, in rat heart-derived cells, GA uptake is inhibited by the addition of alpha-cyano-4-hydroxycinnamate or 4,4′-dibenzamidestilbene-2,2′-disulfonate, inhibitors of MCT1 and MCT4. The mean Km value (± SE of the number of experiments shown) for GA uptake in hepatocytes isolated from starved rats was 26.6 ± 9.2 mM. The proton-bound isoform MCT1–4 plays an essential role in the transport of monocarboxylic acids, such as lactate, pyruvate, and keto acid, and is widely distributed in many tissues. Therefore, the cellular uptake of GA in the body is relatively unrestricted in the physiological concentration range.

GA Excretion

When 101 healthy subjects in the USA were allowed to follow a self-selected diet, the mean urinary excretion (± SD) of GA/creatinine was 40.5 ± 11.5 mmol/mol for men ($n = 54$) and 49.4 ± 18.6 mmol/mol for women ($n = 47$) ($p = 0.003$). When 11 subjects (5 males and 6 females) consumed 2 diets of low or high protein for 10 days, the urinary glycated creatinine excretion (± SD) after 24 h was 29.9 ± 7.5 mmol/mol in the low-protein group (0.6 g/kg BW/day) and 50.4 ± 12.6 mmol/mol in the high-protein group (1.8 g/kg BW/day) (Holmes et al. 1993). The low-protein diet contained 0% to 13% of total protein from animal sources, whereas the high-protein diet contained 55–65% of animal protein. Therefore, it is presumed that the increase in GA excretion is due to the high protein, especially in animal protein, intake, and catabolism.

GA excretion (molar ratio of GA/creatinine) in childhood was highest during the first 6 months of life (72–425 mmol/mol, 95% confidence interval), especially around 1 month of age. It increased from 4 to 28 days of age and then decreased,

mainly in the first 4 years of life (48–191 mmol/mol at age 5) and in the age group up to 16 years (22–99 mmol/mol at age 16). Urinary GA excretion varied considerably in value, although GA excretion in urine sampled at any time in the afternoon during nonfasting periods was not significantly different from the mean value of GA in urine sampled in early morning fasting between the ages of 5 and 16 years (Leumann et al. 1990). The GA excretion of the individuals mentioned above (mean age, 29.3 years) on a self-selected diet was 57.7 mmol/mol, which is comparable to the value at an age of 16 years.

GA Metabolism

Glyoxylate Detoxification

The metabolic pathway involving GA is understood as glyoxylate-centered metabolic pathways (Fig. 1). Because glyoxylate is highly toxic by itself, glyoxylate is detoxified by three metabolic pathways. The first of which catalyzes the transamination of alanine and glyoxylate to glycine and pyruvate, catalyzed by alanine/glyoxylate aminotransferase 1 (Agxt1), which is localized in the peroxisomes, or Agxt2, which is localized in the mitochondria (Holmes and Assimos 1998). The second pathway is the metabolism of glyoxylate to GA by mitochondrial or cytosolic glyoxylate reductase/hydroxypyruvate reductase (GRHPR). The third pathway is the metabolism of glyoxylate to oxalate by lactate dehydrogenase (LDH) in the cytoplasm. Genetic abnormalities in Agxt1 (primary hyperoxaluria type 1, PH1) and

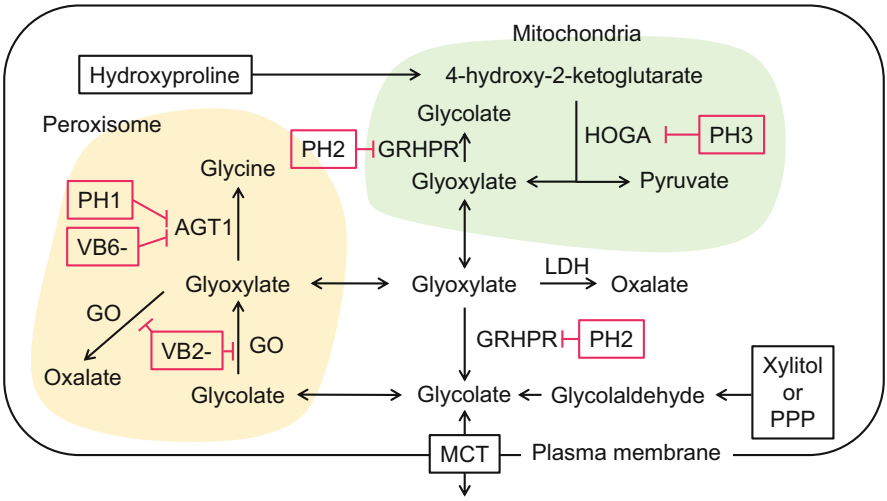

Fig. 1 Glycolate metabolism in the liver. *AGT1* alanine/glyoxylate aminotransferase, *GO* glycolate oxidase, *GRHPR* glyoxylate reductase/hydroxypyruvate reductase, *HOGA1* 4-hydroxy-2-oxoglutarate aldolase, *LDH* lactate dehydrogenase, *PH* primary hyperoxaluria, *PPP* pentose phosphate pathway, *VB* vitamin B

Grhpr (primary hyperoxaluria type 2, PH2), which inhibit the glyoxylate catabolic pathway, increase the rate of oxalate catabolism production and subsequently cause hyperoxaluria. Hyperoxaluria is a significant cause of kidney stones, and approximately 70%–80% of stones are composed of calcium oxalate or a mixture of calcium oxalate and calcium phosphate. There is compelling evidence that most of the glyoxylate and GA produced in hepatocytes are successfully metabolized to glycine in peroxisomes. Because oxalic acid is also synthesized from GA in isolated peroxisomes, it is assumed that GA oxidase (GO) catalyzes the metabolism of glyoxylate to oxalic acid. This point needs to be further investigated in the future.

Glycolate Oxidase (GO)

The enzyme GO [EC 1.1.3.1], also known as (L-2-)hydroxy-acid oxidase, is widely conserved in intestinal bacteria, plants, and mammals (Clagett et al. 1949; Jones et al. 2000; Lee et al. 2019). In humans, GO is a liver-specific enzyme encoded by the hydroxyacid oxidase-1 gene (Hao1) that catalyzes the conversion of GA to glyoxylate in the peroxisome (Martin-Higueras et al. 2016). GO belongs to the flavoenzyme family, which uses riboflavin metabolites such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as cofactors. Isolated GO from hog kidneys uses GA rather than DL-glyceric acid, alpha-hydroxybutyric acid, and DL-lactic acid, with the Km values for GA and FMN being 0.30 and 0.71 mM, respectively (Tokushige and Sizer 1967). An analysis of the binding affinities of FMN and FAD to GO of nine plant species from seven families showed that FMN has 1.4–3.4 times higher binding affinity than FAD and riboflavin did not bind to GO (Wang et al. 2004). The only difference between FMN or FAD and riboflavin is the substitution of inorganic phosphate for 5′-OH. Additionally, GO activity is inhibited by inorganic phosphate, indicating that the 5′-phosphate moiety of FMN and FAD plays an important role in the recognition and binding of apoGO and its cofactors.

Several approaches have been reported to prevent kidney stones by reducing hyperoxaluria, including medical treatment and dietary therapy. One approach is to reduce the activity of GO. Some studies have shown that suppressing GO expression or activity in Agxt1-deficient mice ameliorates hyperoxaluria (Li et al. 2016; Martin-Higueras et al. 2016; Liebow et al. 2017), and Hao1 knockout mice showed increased urinary GA levels without renal damage or other phenotypic effects. Furthermore, Hao1 deficiency in humans causes asymptomatic hyperglycolic aciduria (Frishberg et al. 2014). A healthy adult with a Hao1 knockout showed that her plasma GA level was 12 times and urinary GA was six times the upper limit observed in healthy reference individuals ($n = 67$). These findings are consistent with the concept that GA is highly water-soluble and is excreted by the kidney without pathological consequences. Furthermore, in Agxt1 knockout mice treated with Hao1 siRNA, changes in serum and urinary GA concentration were inversely correlated with GO mRNA expression in the liver. This suggests that GA could be used as a pharmacodynamic marker to assess GO activity using a simple, noninvasive procedure to monitor GA levels in urine and plasma.

Vitamin B2 and GA Metabolism

Vitamin B2, also known as riboflavin, is an essential nutrient in the intermediate metabolism of carbohydrates, amino acids, and fats and supports cellular antioxidant activity. This vitamin fulfills these functions by being metabolized into a coenzyme form. Free riboflavin is converted to the coenzyme form in two steps. Firstly, it is converted to riboflavin 5′-phosphate by ATP-dependent phosphorylation by cytosolic flavokinase (also called riboflavin kinase). FMN is then converted by FAD synthase to FAD. Riboflavin with these phosphate groups acts as a cofactor for many enzymes.

A recent study found that mice fed a VB2-deficient diet for 2 weeks showed increased plasma GA concentrations, and a 2-week VB2-deficient diet reduced GO activity in the liver, the main organ of GO expression, to 47%. The decrease in GO activity and the increase in plasma GA concentration observed with the VB2-deficient diet were ameliorated by supplementation of VB2. Two weeks of VB2-deficient diet was not accompanied by a significant decrease in plasma or blood VB2 concentration. However, plasma VB2 concentration and plasma GA concentration showed a significant negative correlation ($R^2 = 0.42$, $p = 0.03$) (Uebanso et al. 2020). The GO activity of the liver was negatively correlated with the concentration of GA in plasma ($R^2 = 0.45$, $p = 0.01$). A more recent study found that endogenous oxalate production was altered by different VB2 nutritional statuses. We found that feeding a VB2-deficient diet to a mouse model of hyperoxaluria suppressed GO activity in the liver by 51% and urinary excretion of oxalate/creatine by 42% (Uebanso et al. 2021). This ability of VB2 to reduce GO activity suggests that VB2 may be a new element among dietary factors to prevent hyperoxaluria and renal stone disease.

VB2 is produced by several bacteria, including lactobacilli in the gut, and the host expresses the VB2 transporter in the distal gut. However, there are only estimates of VB2 that may be produced and supplied to the host by the gut microbiota (Magnúsdóttir et al. 2015). In a study reported by Magnusdottir et al., only 2.8% of the dietary reference intake was considered to be supplied by the gut microbiota in humans. Our previous study revealed that when mice were given antibiotics to disrupt their gut microbiota, plasma GA levels increased rapidly when fed with a VB2-deficient diet. The time course of the changes in urinary GA concentrations during vitamin B-deficient diet intake showed that gut microbiota disruption accelerated the increase in urinary excretion of GA from 7 to 2. These results suggest that when dietary VB2 intake is inadequate, the supply of VB2 from intestinal bacteria helps in hosting VB2 homeostasis (Fig. 2).

Agxt1

AGT1 (EC 2.6.1.44) is encoded by the Agxt1 gene and is found in the peroxisomes of the human liver. AGT1 is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes L-alanine and glyoxylate pairs to pyruvate and glycine in peroxisomes. As

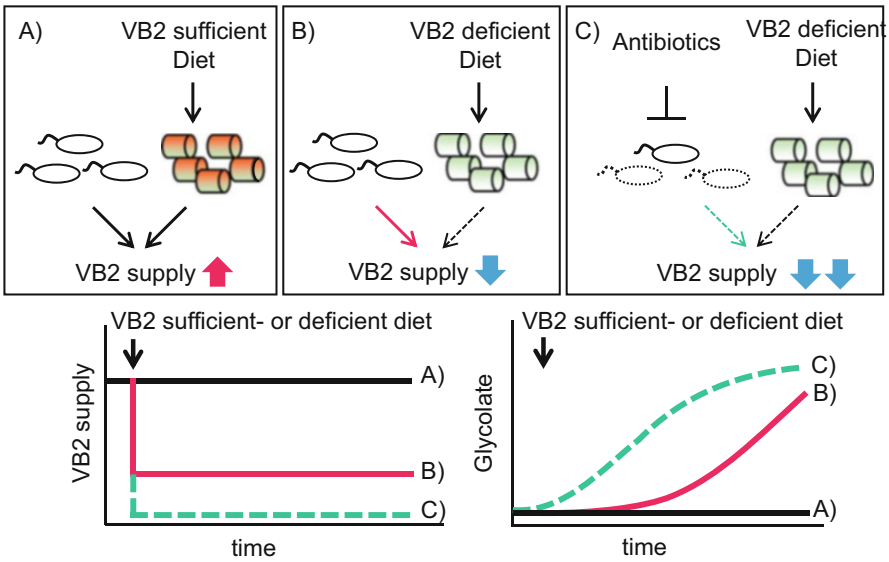

Fig. 2 Effect of the disruption of vitamin B2 supply from diet and gut on glycolate level. (**a**) VB2-sufficient diet and normal gut microbiota supply adequate VB2 to host. In this case, the glycolate level is not increased. (**b** and **c**) In the case when mice are fed VB2-deficient diet, gut bacteria supply some VB2 to host and delay VB2 deficiency-induced accumulation of GA

with many other aminotransferases, in the first half of the reaction, a PLP-type enzyme (AGT1-PLP) reacts with L-alanine to produce pyruvate and a pyridoxamine 5′-phosphate enzyme (AGT1-PMP). Next, in the latter reaction, AGT1-PMP binds glyoxylate and converts it to glycine, regenerating AGT1-PLP. It has been shown that the binding affinity of this enzyme for PMP is at least three times higher than that for PLP (Ishikawa et al. 1996). Recombinant holoAGT1 binds 2 moles of the PLP dimer, and the binding of PLP to the apoenzyme increases linearly with PLP concentration in the range of 100–840 μM. AGT1 is highly specific for the reaction of glyoxylate to glycine: (i) the Km of AGT1 for glyoxylate is as low as 0.01 mM (Cellini et al. 2007), (ii) the overall transamination kcat of the alanine/glyoxylate pair is approximately 100-fold higher than that of the glycine/pyruvate pair, and (iii) unlike pyruvate, glyoxylate does not inhibit the AGT1 reaction up to at least 100 mM. This clearly indicates that in vivo, AGT1 is only devoted to metabolizing glyoxylate to glycine. Therefore, loss-of-function Agxt1 mutations that result in the loss of the potent detoxification of glyoxylate by AGT1 are responsible for primary hyperoxaluria.

Vitamin B6 and GA Metabolism

There are three natural forms of VB6: pyridoxine (Pn), pyridoxal (Pl), and pyridoxamine (Pm), each of which performs its biological function by being metabolized to the common coenzyme pyridoxal phosphate (PLP). Pn, Pl, and Pm are

phosphorylated by pyridoxal phosphatase, and then, pyridoxamine phosphate oxidase converts Pn and Pm to the coenzyme PLP. This enzyme requires FMN as a cofactor. Therefore, VB6 and VB2 metabolism forms a complex. Enzymes containing PLP are ubiquitous, performing essential reactions in the metabolism of amino acids and amines.

The excretion of GA was shown to increase in rats fed a VB6-deficient diet, a coenzyme of AGT1, for 3 or 4 weeks (Nishijima et al. 2006; Teerajetgul et al. 2008). Agxt1 mRNA expression in the liver was lower in VB6-deficient diet-fed rats, suggesting that VB6 deprivation may regulate both mRNA expression and the activity of AGT1. By contrast, another study showed that in mice fed a VB6-deficient diet for 2 weeks, no increase in GA concentration was observed and VB6 supplementation of the four vitamin B (B2, B6, B9, and B12)-deficient diet did not affect GA concentration. At this time, the concentration of VB6 in plasma decreased to 10% of that in the control group, indicating that VB6 deficiency does not seem to be related to changes in GA concentration in mice. These results suggest that the glyoxylate and GA metabolic pathways may differ between rats and mice. Indeed, the pharmacokinetics of ethylene glycol, involved in the glyoxylate metabolism pathway, do differ between rats and mice (Frantz et al. 1996). Tissue distribution and metabolic kinetics were studied in ^{14}C-labeled ethylene glycol Sprague-Dawley rats and CD-1 mice after intravenous, oral, and dermal administration. ^{14}C-labeled ethylene glycol, GA, and oxalic acid accounted for most of the detectable radioactivity in urine from all routes of administration in rats. Alternatively, a similar amount of GA but no oxalic acid was detected in urine samples from intravenous and oral doses in mice. These observations suggest that the amount of glyoxylate metabolized to oxalic acid is higher in rats than in mice and that the role of AGT1 in glyoxylate detoxification is more significant.

In 13 of 16 human PH1 patients, pyridoxine administration reduces the urinary oxalate load by approximately 30% (van Woerden et al. 2004). However, the effects of VB6 on kidney stone formation have not been consistent in the subject without PH (Taylor et al. 2004; Ferraro et al. 2017). The relation between the intake of VB6 and the risk of symptomatic kidney stones was prospectively studied in a cohort of 85,557 women with no history of kidney stones (Curhan et al. 1999). A total of 1078 incident cases of kidney stones were documented during the 14-year follow-up period. After adjusting for other dietary factors, the relative risk of incident stone formation for women in the highest category of B6 intake ($\geq$40 mg/d) compared with the lowest category ($<$3 mg/d) was 0.66 (95% confidence interval, 0.44 to 0.98). A high intake of VB6, based on the supplement intake, was inversely associated with the risk of stone formation. Analysis of three follow-up studies, including Health Professionals Follow-up Study (n = 42,919 men), Nurses' Health Study I (n = 60,003 older women), and Nurses' Health Study II (n = 90,629 younger women), which have a total of 6576 incident kidney stones during 3,316,846 person-years of follow-up, showed that there was no association between the intake of VB6 and incident stones (Ferraro et al. 2018). These results suggest that pyridoxine is more effective in conditions where AGT1 activity is reduced.

Factors Influencing GA Metabolism

In addition to B vitamins, there are other factors known to affect GA metabolism. The effect of hereditary hyperoxaluria on GA metabolism is controversial. Buchalski et al. showed increased urine GA levels in both male and female Agxt1 KO mice but not in Grhpr KO mice (Table 1). According to a survey of a small group of people, patients with PH1 showed higher plasma and urine GA concentrations than those of control subjects (Fargue et al. 2018). By contrast, another report showed that Agxt1 knockout mice exhibit hyperoxaluria but no increase in urinary GA concentration (Martin-Higueras et al. 2016; Zabaleta et al. 2018). These differences might come from other factors that influence GA metabolisms, such as diet and age. In dietary

Table 1 Factors influencing glycolate excretion

Factors	Changes in GA metabolism	Urinary or Plasma GA	Species	Reference
PH1	Reduced AGT1 activity	↑ → or ↑	Human Mouse	(Fargue et al. 2018) (Martin-Higueras, Luis-Lima and Salido 2016; Zabaleta et al. 2018; Buchalski et al. 2020)
PH2	Reduced GRHPR activity	→ →	Human Mouse	(Fargue et al. 2018) (Buchalski et al. 2020)
PH3	Reduced HOGA1 activity	↓	Human	(Fargue et al. 2018)
Vitamin B2 deficiency	Reduced GO activity	↑	Mouse	(Uebanso et al. 2020)
Vitamin B6 deficiency	Reduced AGT1 activity	↑	Rat	(Nishijima et al. 2006; Teerajetgul et al. 2008)
Hyp intake	Increased substrate Increased GO activity	↑ ↑	Human Mouse	(Fargue et al. 2018) (Uebanso et al. 2021)
Xylitol intake	Increased substrate from PPP?	↑	Human	(James et al. 1982; Holmes and Assimos 1998)
Diabetes	Increased substrate from PPP?	↑(glyoxylate) ↑(glyoxylate)	Mouse Human	(Giesbertz et al. 2015) (Padberg et al. 2014)
Aging	Reduced GO activity	?	Mouse	(Uebanso et al. 2021)

AGT1 alanine/glyoxylate aminotransferase 1, *GA* glycolate, *GO* glycolate oxidase, *GRHPR* glyoxylate reductase/hydroxypyruvate reductase, *HOGA* 4-hydroxy-2-oxoglutarate aldolase *Hyp* hydroxyproline, *PH* primary hyperoxaluria, *PPP* pentose phosphate pathway.? further study is needed

factors, glyoxylate is also produced in the mitochondria through the metabolism of 4-Hyp in hepatocytes and proximal renal tubules (Fig. 1). The amount of Hyp degraded per day is 300–400 mg, and in Hyp or collagen catabolism, 4-hydroxy-2-ketoglutarate is cleaved by the mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase (HOGA1, which is the gene responsible for PH3) to pyruvate and glyoxylate. Excessive amounts of Hyp and glyoxylate lead to relative increases in the production of GA and oxalate (Table 1). Hyp is derived from meat and gelatin in the diet and from normal turnover of collagen. In a study of rats, Takayama et al. demonstrated increased excretion of oxalate and GA in animals given 630 mg (4.8 mmol) of Hyp in 2 mL of water after 48 h of fasting (Takayama et al. 2003). A prior injection with glucagon, demonstrated to induce mitochondrial AGT1, reduced the response to Hyp. It was estimated that the normal breakdown of collagen contributes 20% to 50% of excreted GA produced endogenously (Knight and Holmes 2005; Knight et al. 2006). In our previous study, we found that the GO activity in the liver was also upregulated in Hyp supplementation. Therefore, increased supply of substrate could upregulate GO activity and influence GA metabolism.

Age is also reported to be inversely associated with urinary oxalate levels (Taylor and Curhan 2008). In our previous study, we investigated age-related changes in GO activity. Young mice (12 weeks old) had higher hepatic GO activities than those of old mice (over 24 weeks old) fed a chow diet. GO activity was reduced in an age-dependent manner in both lean and obese mice (Table 1). This change was diet independent, and further studies are needed to understand the detail regulatory mechanism of GO activity by aging.

Recently, obesity and metabolic syndrome have received much attention as causes of secondary hyperoxaluria, nephropathy, and kidney stones in the absence of genetic abnormalities. It has been reported that there is a positive correlation between urinary oxalate concentration and the increase in body mass seen in obesity and metabolic syndrome (Lemann et al. 1996; Taylor and Curhan 2008). Approximately half of human oxalate is supplied by the diet, and the other half is endogenously synthesized in the body from glyoxylate. Current evidence suggests that obesity with hyperoxaluria, mainly caused by increased oxalate intake due to overeating, may lead to increased paracellular absorption from the intestine and decreased oxalate excretion from the intestine to inflammation (Amin et al. 2018; Bashir et al. 2019). Our results showed that obese mice have low GO activity and that urinary oxalate excretion is not inhibited by feeding a VB2-deficient diet. These results suggest that endogenous oxalate production may not be a significant contributor to hyperoxaluria in our obesity model. In support of this result, to the best of our knowledge, there is no report of increased de novo oxalate production in obese mice. Glyoxylate levels were sixfold higher in plasma from obesity-induced diabetic model (db/db) mice (Giesbertz et al. 2015). Metabolomic profiling of human plasma showed that increased glyoxylate levels could be a potential metabolite marker of diabetes mellitus (Padberg et al. 2014). Indeed, diabetes mellitus was independently associated with higher urinary oxalate excretion, after adjustment for a number of variables including

medications, body mass index, age, race, sex, and laboratory tests (Waikar et al. 2019). A reason for the increase in plasma glyoxylate levels in diabetes mellitus is thought to be the increase in the amount of glucose entering the pentose phosphate pathway (PPP). The metabolism of xylulose-5-phosphate, a metabolite of the PPP, to glycolaldehyde, a precursor metabolite of glyoxylate, has been suggested (Fig. 1) (Table 1). Indeed, 20-g xylitol ingestion increased oxalate excretion in the subject (the average BW is 64.4 kg ± 0.3 g/kg BW) (Nguyen et al. 1993). These results support the contribution of xylitol feeding per se or its metabolites to GA and oxalic acid metabolism. As such, GO-targeted therapies, such as a VB2-deficient diet, are more effective when the GA metabolism is activated.

Markers in VB2 and VB6 Deficiency and Applications to Prognosis

Because the deficiency of VB2 and VB6 is a risk factor for various diseases and is involved in the activation of other vitamins, it is essential to assess properly the nutritional status of these. Several biomarkers for VB2 and B6 deficiency have been reported so far. Several clinical and biochemical endpoints have been used to assess VB2 status (Hoey et al. 2009). Among them, urinary VB2 concentration and erythrocyte glutathione reductase activating factor (EGRAC) are currently used as biomarkers to reflect the nutritional status of VB2 (Mushtaq et al. 2009). In an experiment, urinary VB2 concentration rapidly and dramatically decreased within 2 days after administering a VB2-deficient diet in mice. Alternatively, urinary GA concentration increased 7 days after VB2-deficient diet administration. Thus, the change in urinary GA concentration occurred more slowly than that in urinary VB2 concentration, reflecting the degree of VB2 sufficiency in the liver because the change in GA concentration involved changes in the enzyme activity of EGRAC. In contrast to EGRAC, urinary GA concentrations can be easily measured. Therefore, measuring both VB2 and GA in urine as dual biomarkers of VB2 nutritional status can provide information on the degree of dietary VB2 intake and host VB2 sufficiency/deficiency status. Additionally, because the disruption of the intestinal microflora by antibiotics administration hastens the increase in GA excretion due to VB2 deficiency, the ability of the intestinal bacteria to supply VB2 can be assessed.

The most common method of assessing long-term VB6 status is the measurement of plasma PLP (Ueland et al. 2015). Plasma PLP is a good reflection of the amount of VB6 stored in body tissues. Alternatively, 4-pyridoxic acid, a catabolite formed from Pl, is more sensitive than PLP, although it is affected by other confounding factors, suggesting its potential as a complementary and short-term marker of VB6 status. Plasma PLP and 4-pyridoxic acid can be analyzed directly using high-performance liquid chromatography. It is known that GA is increased in rats during VB6 deficiency, but data and kinetic information in humans are lacking. Urinary and plasma GA concentrations can be measured directly by capillary electrophoresis mass spectrometry and are awaiting further investigation.

Applications to Prognosis

In this chapter, we reviewed reports in mice but not in human that glycolic acid is associated with nutritional status of vitamins B2 and B6. In humans, SNPs in the intron region of GO have been reported to be associated with the development of kidney stone. Whole-genome single nucleotide polymorphism (SNP) genotyping results showed significant differences in allele frequencies of 11 SNPs located in the intron region of the Hao1 gene encoding GO between nephrolithiasis patients and healthy controls (Rungroj et al. 2014). Although the details of the effects of these SNPs on human GA metabolism are unknown, their genomic location makes it highly likely that they will affect the expression levels and activity of GO. The relationship between GO activity and GA metabolism and the nutritional status of vitamins B2 and B6 requires further investigation.

Applications to Other Diseases or Conditions

In general, glyoxylic acid is endogenously favored from hydroxyproline and collagen catabolism, but it is also synthesized as a byproduct of glucose metabolism by the pentose phosphate pathway. During hyperglycemia, the metabolic flux of the pentose phosphate pathway is increased, resulting in an increase in glycolic acid and glyoxylic acid. Therefore, these metabolites may be used as indicators of activation of the pentose phosphate pathway in hyperglycemia and diabetes.

Mini-Dictionary

db/db mouse. Genetic mouse models of obesity and type 2 diabetes with mutation in leptin receptor.

Kidney stone. Also known as kidney stones, nephrolithiasis, or urinary stones, are hard deposits made of minerals and salts that form inside the kidneys.

Monocarboxylic acid transporters (MCTs). Transporters of monocarboxylic acids (lactate, acetate, ketone bodies, etc.), and 14 subtypes (MCT1 to MCT14) have now been identified.

Pentose phosphate pathway (PPP). One of the branches of the glycolytic system, and it is essential for DNA synthesis and maintenance of the intracellular redox state.

Xylitol. A type of sugar alcohol synthesized from xylose and it is known as a natural substitute sweetener.

Key Facts of Xylitol Metabolism

- The sugar alcohol xylitol inhibits the growth of some bacterial species including *Streptococcus mutans*, and it is used as a food additive to prevent caries.

- Xylitol comestible products (e.g., gums and candies) have been commercially available to the general public.
- Some infants are given xylitol tablets for dental health (the dose is up to 200 mg/kg body weight/day).
- Xylitol intake of up to 200 mg/kg body weight/day significantly alters the intestinal microbiota of mice.
- Dietary xylitol, metabolized into D-xylulose-5-phosphate, activates the carbohydrate response element binding protein (ChREBP) and lipogenesis.

Summary Points

- GA is readily absorbed in large amounts from the gastrointestinal tract, and the cellular uptake of GA in the body is relatively unrestricted in the physiological concentration range.
- Glyoxylate is highly toxic by itself; glyoxylate is detoxified by three metabolic pathways by Agxt1, GRHPR, GO, or LDH.
- Several factors, including diet, age, and diabetes, influence GO activity.
- Vitamins B2 and B6 influence the metabolism of GA through the regulation of GO and AGT1 activity, respectively.
- GA may be a marker of VB2 or VB6 deficiency, and further studies in humans are needed.

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Assays for Carotenoids: Linking Breastmilk and Maternal Intakes

13

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Abstract

Humans are not able to synthesize carotenoids and rely on their dietary intake mainly with vegetables and fruit, algae, and some animal products. The major dietary and plasma carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene, and β-cryptoxanthin. Carotenoids are transferred to the mammary gland and breastmilk. Maternal dietary intake, geographical locations, age, nutritional status, premature birth, and circadian and within-feeding variations may affect breastmilk carotenoid levels. Those factors, especially circadian and within-feeding variations, should be included in studies assessing breastmilk carotenoids. During handling samples should be protected against oxygen, light, and temperature and stored in a −80 °C. The most recommended for the determination of carotenoids in breastmilk is a normal- or reversed-phase HPLC/UHPLC after specific sample preparation (saponification, hydrolyzation, and extraction). The most recommended method of nutritional data collection is at least a 3-day food record along with FFQ. Additionally, national databases of food carotenoids need to be developed to accurately estimate their intake.

Keywords

α-Carotene · β-Carotene · Breastmilk · β-Cryptoxanthin · Carotenoids · High-performance liquid chromatography (HPLC) · Lutein · Lycopene · Maternal diet · Nutritional assessment · Zeaxanthin

Abbreviations

α-CA	α-carotene
α-CR	α-cryptoxanthin
β-CA	β-carotene
β-CR	β-cryptoxanthin
BMI	body mass index
CD36	cluster of differentiation 36
FFQ	food frequency questionnaire
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
L	lutein
L + Z	lutein+zeaxanthin
LDL	low-density lipoprotein
LY	lycopene
NPC1L1	Niemann-Pick C1-Like 1
NS	nonsignificant
RAE	retinol activity equivalents
SR-BI	scavenger receptor class B type 1
UHPLC	ultra-high-performance liquid chromatography
UPLC	ultra-performance liquid chromatography
USDA	United States Department of Agriculture
Z	zeaxanthin

Introduction

Breastfeeding is a gold standard in infant nutrition: exclusive breastfeeding is recommended up to 6 months of infant life, followed by further breastfeeding up to 2 years or more (WHO 2003; Fewtrell et al. 2017). For child, breastmilk is not only a source of energy and all essential nutrients but also thousands of different bioactive compounds, including immune and growth factors, hormones, and phytochemicals (Miller et al. 2013; Samuel et al. 2020; Ríos et al. 2021). Breastmilk compositions vary and change dynamically during the course of lactation to fit to changing nutritional requirements and developmental needs of the child (Miller et al. 2013; Samuel et al. 2020). Those changes are related to the maternal, infant, and physiological factors, including maternal diet and nutritional status or lactation stage and the phase of single nursing (Miller et al. 2013; Samuel et al. 2020). Breastmilk always is the best and adequate source of nourishment for the infant, also in case when maternal diet and nutritional status are suboptimal (Butte et al. 2001). Breastmilk is a dynamic fluid that can vary its composition depending on individually multiple factors, including maternal diet and food intake (Miller et al. 2013; Ríos et al. 2021). There are several nutrients and bioactive substances in breastmilk that are diet dependent, including vitamins C, B_6, and B_{12}, choline, iodine, selenium, and phytochemicals such as carotenoids (Tsopmo 2018; Ríos et al. 2021). Carotenoids are lipophilic pigment synthesized by plants and some microorganisms (photosynthetic bacteria, some species of archaea and fungi, algae). As mammals, humans are not able to produce carotenoids and rely on their dietary intake, mainly with vegetables and fruits, and some seafood (Böhm et al. 2021). We can distinguish two classes of carotenoids: carotenes (e.g., β-carotene, α-carotene, lycopene) and more polar xanthophylls (e.g., lutein and zeaxanthin (L + Z), β-cryptoxanthin, astaxanthin) (Krinsky and Johnson 2005; Jomova and Valko 2013; Reboul 2019). More than 650 different carotenoids are present in nature, and in the human diet around 50–100 of them are found, whereas in human serum only 6 compounds (β-carotene, α-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin) make up more than 90% of total serum carotenoids (Fig. 1) (Krinsky and Johnson 2005; Rao and Rao 2007; Eggersdorfer and Wyss 2018). Those six carotenoids are also the most common in breastmilk samples due to plasma-breastmilk transport (Schweigert et al. 2004; Meneses and Trugo 2005; de Azeredo and Trugo 2008; Lipkie et al. 2015; Zielinska et al. 2017a; Ríos et al. 2021).

Carotenoid's Absorption and Transport

After ingestion carotenoids are released from the food matrix and diluted in the lipid phase prior to the absorption in the small intestine. Like other lipids, carotenoids are absorbed via passive diffusion or active transport via, e.g., SR-BI, CD36, and NPC1L1 transporters (Krinsky and Johnson 2005; Reboul 2019; Böhm et al. 2021). In the enterocytes carotenoids are incorporated into chylomicrons and secreted into the lymph and further transported to the liver (Krinsky and Johnson 2005; Böhm et al. 2021). From the liver, carotenoids are released to the serum into

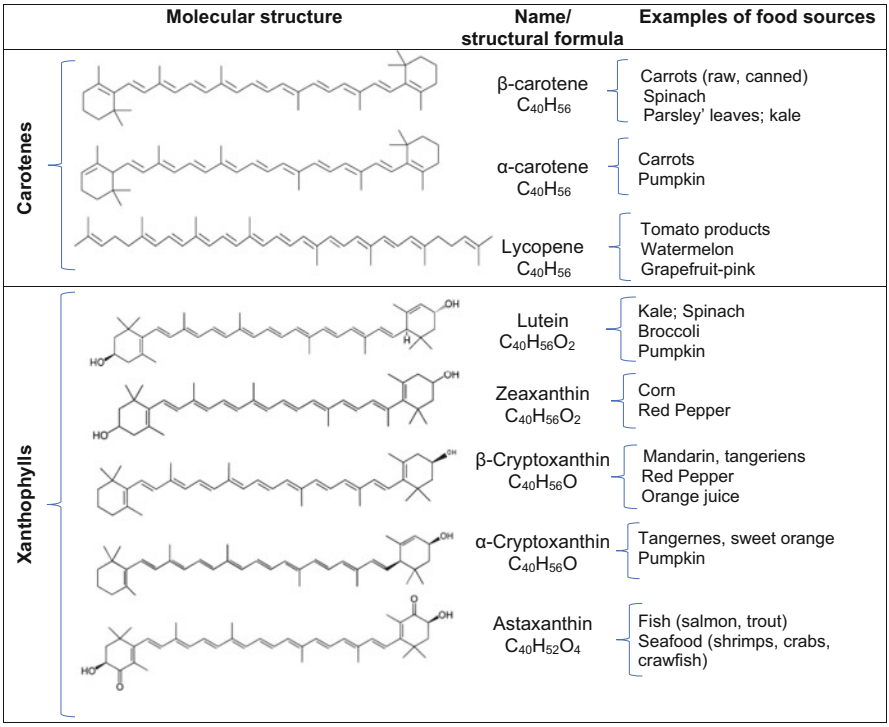

Fig. 1 The chemical structures of the selected carotenoids and their major dietary sources

lipoproteins. Interestingly, carotenes are mainly incorporated into low-density lipoprotein (LDL), whereas xanthophylls are incorporated equally between LDL and high-density lipoproteins (HDL). Moreover, due to different polar characteristics, carotenes are present in the lipoprotein core, whereas xanthophylls are located on its surface, which may explain its equal distribution between LDL and HDL fractions (Krinsky and Johnson 2005; Machado et al. 2019). Carotenoids are accumulated in several organs and tissues, especially the liver, adrenal gland, skin, lung, adipose tissue, brain, retina, prostate, breast, and breastmilk (Böhm et al. 2021). However, the distribution of carotenoids in human organs shows a high specificity depending on the carotenoid type.

Carotenoid Transport into the Breastmilk

In breastmilk, carotenoids are present in 10–120 times lower concentrations than plasma. However, the different serum to breastmilk carotenoid ratio may be observed depending on their polarity (polar xanthophylls have a higher ratio than less polar carotenes) (Meneses and Trugo 2005; Lipkie et al. 2015; Zielinska et al. 2017a; Böhm et al. 2021). Moreover, when the changes in carotenoid levels in

breastmilk were observed, their serum levels were intact (Schweigert et al. 2004; Machado et al. 2019). This indicates that despite the similar fate of carotenoids and fat in the intestine, carotenoids may be transported into breastmilk differentially, independent from fat (Macias and Schweighert 2001; Zielinska et al. 2017a). Possible mechanisms of carotenoid transport from plasma into breastmilk cover intracellular transport and preferential uptake by lipoproteins, especially the HDL fraction (Zielinska et al. 2017a; Machado et al. 2019).

Breastmilk Composition

Breastmilk carotenoids are part of complex and variable lipid fractions (Duan et al. 2019; Samuel et al. 2020), and their profile changes differently compared to plasma (Macias and Schweighert 2001). It is well known that breastmilk fat concentration changes throughout lactation (Samuel et al. 2020). The lowest amount of fat is present in colostrum, but then increases rapidly, remains relatively stable in the mature milk in the first year of lactation, but then increases again (Mitoulas et al. 2002; Miller et al. 2013; Czosnykoska-Łukacka et al. 2018; Samuel et al. 2020). Breastmilk fat levels are also regulated by circadian variations – the highest concentration is observed in the morning and evening (Italianer et al. 2020; Samuel et al. 2020). Another factor determining its concentrations is within-feeding variation (Samuel et al. 2020). Hindmilk (milk at the beginning of single feeding or prefeed milk) contains much lower levels of fat than foremilk (milk at the end of single feeding or postfeed milk) (Khan et al. 2013; Samuel et al. 2020). On the contrary, breastmilk fat levels did not vary between breasts (Mitoulas et al. 2002; Pines et al. 2016) or regarding the volume of produced breastmilk and emptying the breast at the previous feeding (Samuel et al. 2020). Method of breastmilk expression (manual vs. electrically by breast pump) may also influence fat level in milk (Samuel et al. 2020). Results about the relation to the number of breastfeeding or intervals between feeding are inconclusive (Khan et al. 2013; Samuel et al. 2020).

Breastmilk Carotenoids

In the last two decades, breastmilk carotenoids were widely investigated (Zielinska et al. 2017a; Ríos et al. 2021). In many studies, predominant breastmilk carotenoid is lutein (or lutein+zeaxanthin) (Macias et al. 2001; Ríos et al. 2017; Wu et al. 2020) or β-carotene (Machado et al. 2019). Interestingly, a recent study reported for the first time in human colostrum free apocarotenoids and its esters, carotenoid metabolites derived from oxidative or enzymatic cleavage (Zoccali et al., 2020). As shown in Table 1 carotenoid concentrations change within the course of lactation, with the highest level in the colostrum, and decrease especially between the 2nd and 4th weeks of lactation (Jackson et al. 1998; Sommerburg et al. 2000; Macias et al. 2001; Schweigert et al. 2004; Cena et al. 2009; Song et al. 2013; Ríos et al. 2017; Xue et al. 2017; Xavier et al. 2018; Machado et al. 2019; Xavier et al. 2019; Wu et al. 2020;

Table 1 Breastmilk carotenoids according to lactation stage

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
Cuba $N = 21$ Macias and Schweigert (2001)	10–12 mL of foremilk (morning); manual expression	HPLC nmol/L	β-CA: 125.7 ± 6.37 L: 67.9 ± 44.9 Z: 9.7 ± 6.7 LY: 137.3 ± 86.1 β-CR: 61.1 ± 66.6	β-CA: 44.2 ± 34.1 L: 44.5 ± 36.1 Z: 8.6 ± 5.5 LY: 44.2 ± 34.1 β-CR: 24.8 ± 22.4	β-CA: 36.2 ± 17.2 L: 27.3 ± 16.4 Z: 7.9 ± 7.7 LY: 18.8 ± 2.7 β-CR: 16.6 ± 12.7
Germany $N = 21$ Schweigert et al. (2004)	Total volume from one breast; no data	HPLC nmol/L	β-CA: 423.4 ± 326.6 L: 164.0 ± 84.9 Z: 33.2 ± 84.9 LY: 508.9 ± 421.7 β-CR: 238.8 ± 156.1	–	β-CA: 78.2 ± 46.2 L: 88.1 ± 37.8 Z: 19.5 ± 10.2 LY: 59.8 ± 38.9 β-CR: 60.6 ± 36.7
Italy $N = 21$ Cena et al. (2009)	5–6 mL of milk; breast pump	HPLC nmol/L	L: 280 ± 220	–	L: 110 ± 50
USA $N = 17$ Song et al. (2013)	Total volume from one breast at 10 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 164.3 ± 25.2 α-CA: 59.0 ± 13.5 L: 121.2 ± 20.9 Z: 46.3 ± 5.4 LY: 119.9 ± 18.9 β-CR: 57.4 ± 10.7 α-CR: 57.4 ± 10.7	β-CA: 88.0 ± 23.3 α-CA: 23.2 ± 4.8 L: 56.4 ± 6.8 Z: 21.4 ± 2.5 LY: 49.5 ± 6.4 β-CR: 24.8 ± 4.4 α-CR: 13.5 ± 2.0
USA $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 71.6 ± 56.7 α-CA: 20.2 ± 10.7 L: 125.5 ± 80.1 Z: 35.5 ± 19.5 LY: 75.2 ± 37.1 β-CR: 35.5 ± 27.1	β-CA: 67.4 ± 54.8 α-CA: 18.6 ± 10.8 L: 89.0 ± 45.7 Z: 26.8 ± 15.1 LY: 55.9 ± 33.2 β-CR: 31.2 ± 21.4

(continued)

Table 1 (continued)

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
				α-CR: 25.2 ± 11.0	α-CR: 21.1 ± 9.6
Mexico $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 62.6 ± 30.4 α-CA: 22.2 ± 12.2 L: 166.0 ± 114.9 Z: 50.4 ± 33.1 LY: 60.3 ± 42.7 β-CR: 72.5 ± 61.1 α-CR: 28.0 ± 14.2	β-CA: 40.5 ± 16.5 α-CA: 14.3 ± 8.1 L: 103.2 ± 63.3 Z: 39.6 ± 33.4 LY: 39.7 ± 22.8 β-CR: 43.7 ± 32.2 α-CR: 20.2 ± 13.2
China $N = 20$ Lipkie et al. (2015)	Total volume from one breast at 9 a.m.–1 p.m.; electric pump	HPLC nmol/L	–	β-CA: 83.0 ± 42.7 α-CA: 18.2 ± 8.5 L: 313.7 ± 161.3 Z: 58.8 ± 34.0 LY: 34.7 ± 19.1 β-CR: 53.6 ± 34.6 α-CR: 39.8 ± 15.8	β-CA: 71.6 ± 30.0 α-CA: 11.3 ± 5.6 L: 206.0 ± 93.9 Z: 37.5 ± 17.6 LY: 13.7 ± 13.2 β-CR: 75.4 ± 68.4 α-CR: 31.3 ± 12.3
China $N = 509$ Xue et al. (2017)	Total volume from one breast at 9–11 a.m.; electric pump	U-HPLC μgl/ 100 mL	β-CA: 8.0 (4.7–15.2) L: 5.7 (2.9–10.2) Z: 1.0 (0.5–1.5) LY: 6.3 (4.0–9.9) β-CR: 6.2 (2.4–12.9)	β-CA: 2.8 (2.0–4.4) L: 7.0 (4.6–10.3) Z: 1.4 (1.0–2.2) LY: 2.5 (1.7–4.3) β-CR: 3.4 (1.7–5.7)	β-CA: 1.7 (1.4–3.1) L: 2.9 (0.9–5.9) Z: 0.8 (0.4–1.5) LY: 1.4 (1.1–2.0) β-CR: 1.7 (1.1–2.6)
Spain $N = 30$ Ríos et al. (2017)	Total volume from one breast; no data	U-HPLC nmol/L	β-CA: 754.8 α-CA: 219.6 L: 486.4 Z: 98.1 LY: 854.3 β-CR: 962.6	–	β-CA: 53.3 α-CA: 14.9 L: 199.8 Z: 64.95 LY: 159.9 β-CR: 145.3

(continued)

Table 1 (continued)

Study group	Milk sample	Analysis method, unit	Carotenoid level by lactation stage		
			Colostrum	Transitional milk	Mature milk
Spain $N = 70$ Xavier et al. (2018)	Total volume from one breast; no data	U-HPLC nM	β + α-CA: 1103 (602.3–2238) L: 486.3 (3229–745.8) Z: 106.4 (73.7–141.4) LY: 1065 (483.0–1846)	–	β + α-CA: 84.8 (59.4–315.0) L: 195.9 (150.3–270.8) Z: 59.9 (38.4–01.0) LY: 192.7 (118.8–221.2)
Brazil $N = 19$ Machado et al. (2019)	10 mL in morning; manual expression	HPLC µmol/L	–	β-CA: 0.17 ± 0.02 α-CA: 0.04 ± 0.01 L + Z: 0.07 ± 0.02 LY: 0.04 ± 0.01	β-CA: 0.14 ± 0.3 α-CA: 0.03 ± 0.01 L + Z: 0.06 ± 0.01 LY: 0.04 ± 0.00
Switzerland $N = 28$ Redeuil et al. (2021)	Total volume from one breast in morning; electric pump	UPLC µgl/ 100 mL	β-CA: 38.9 ± 37.9 L: 7.98 ± 4.93 Z: 1.63 ± 0.94 LY: 25.7 ± 19.1 β-CR: 15.3 ± 8.53	β-CA: 9.18 ± 7.61 L: 7.94 ± 6.33 Z: 2.08 ± 1.38 LY: 5.70 ± 3.40 β-CR: 7.04 ± 5.20	β-CA: 4.53 ± 3.48 L: 4.31 ± 2.52 Z: 1.12 ± 0.68 LY: 2.34 ± 1.2 β-CR: 4.39 ± 4.17
China $N = 42$ Wu et al. (2020)	Total volume from both breasts at 9–11 a.m.; electric pump	HPLC µgl/ 100 mL	β-CA: 11.1 (6.5–23.3) L: 7.1 (5.1–13.0) Z: 2.2 (1.3–3.3) LY: 11.9 (7.1–17.4) β-CR: 3.9 (1.5–7.0)	β-CA: 3.1 (1.8–5.3) L: 9.5 (6.8–13.1) Z: 2.2 (1.3–2.9) LY: 1.3 (0.8–2.5) β-CR: 2.0 (1.3–3.6)	β-CA: 1.8 (1.0–3.1) L: 4.6 (2.9–7.6) Z: 1.1 (0.7–1.9) LY: 0.6 (0.3–1.0) β-CR: 0.8 (0.5–2.1)

α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene, β-CR = β-cryptoxanthin. HPLC = high-performance liquid chromatography. L = lutein. LY = lycopene. L + Z = lutein +zeaxanthin. UHPLC = ultra-high-performance liquid chromatography. UPLC = ultra-performance liquid chromatography. Z = zeaxanthin

Redeuil et al. 2021). Interestingly, this decrease is not observed in serum, which confirms the specific mechanisms of carotenoid transport into milk (Machado et al. 2019). Moreover, a higher drop is observed in carotenes than in the more polar xanthophylls (88.67% vs. 35.92%) (Macias and Schweigert 2001; Schweigert et al. 2004; Wu et al. 2020; Redeuil et al. 2021; Ríos et al. 2021; Sun et al. 2021), as well

as vitamin A non-precursors (Lipkie et al. 2015). Carotenoid levels in mature milk (Table 2) are relatively stable and did not vary across the course of lactation but may be influenced by other factors (Song et al. 2013; Lipkie et al. 2015; Zielinska et al. 2019a). It is not exactly known how factors affecting fat concentrations determine breastmilk carotenoids. Previous studies have shown that carotenoids are probably transported into milk independently to fat (Macias and Schweighert 2001; Schweigert et al. 2004; Machado et al. 2019; Wu et al. 2020) and its content might be not related to fat in mature milk (Duan et al. 2019). However, in one study xanthophylls, but not carotenes, were correlated with milk fat (Wu et al. 2020), while in the study by Giuliano et al. (1994), α- and β-carotene, lycopene, and β-cryptoxanthin were correlated with milk fat. Also, within-feeding variations in breastmilk carotenoid levels were reported (Jackson et al. 1998; Hampel et al. 2017). Jackson et al. (1998) showed that hindmilk contained 25% more total carotenoids than mid- and foremilk. These authors investigated also the diurnal patterns and showed a nonsignificant trend of higher carotenoids at midday (Jackson et al. 1998). In a recent study circadian variation in vitamin A levels (β-carotene + retinol) was also reported but diminished after adjustment for fat (Hampel et al. 2017). The most important determinants of breastmilk carotenoids are geographical location, which is related to differences in dietary patterns and carotenoid intake (Canfield et al. 2003; Jackson and Zimmer 2007; Lipkie et al. 2015; Nguyen et al. 2020; Ríos et al. 2021). Other factors that may affect breastmilk carotenoids are maternal age (de Azeredo and Trugo 2008; Denić et al. 2019), preterm birth (Xavier et al. 2018; Redeuil et al. 2021), and maternal nutritional status (BMI; overweight or obesity) (Panagos et al. 2016; Zielinska et al. 2019a).

Carotenoid Intake During Lactation

In European countries intakes of β-CA, α-CA, L/Z, β-CR, and LY are about 1.5–8.8, 0.25–7.7, 0.78–3.25, 0.17–1.36, and 1.64–8.05 g/d, respectively (Böhm et al. 2021). Currently, only a few studies investigated carotenoid intake during lactation with similar results to those observed in non-lactating individuals (Table 3). Similarly to breastmilk carotenoids, their intake varies between different populations (Meneses and Trugo 2005; Cena et al. 2009, Panagos et al. 2016; Xue et al. 2017; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a). Currently there are no official nutritional recommendations for carotenoid intake (Böhm et al. 2021). Previously, in non-lactating (Böhm et al. 2021) and lactating (Cena et al. 2009) individuals, carotenoid intake was linked to plasma/serum concentrations. Maternal plasma carotenoids were correlated to their breastmilk levels (Schweigert et al. 2004; Meneses and Trugo 2005; Sherry et al. 2014, Lipkie et al. 2015; Machado et al. 2019; Xu et al. 2019). However, results about associations between maternal dietary intake and breastmilk carotenoids are ambiguous: some confirm a strong association (Cena et al. 2009; Panagos et al. 2016; Kim et al. 2018, Zielinska et al. 2019a; Machado et al. 2019), and some did not (Meneses and Trugo 2005; Xue et al. 2017; Xu et al. 2019). Despite the discrepancies in the observational studies, several

Table 2 Breastmilk carotenoids in mature milk

Study group	Milk sample	Analysis method, unit	Specific carotenoids in mature milk				
			β-CA	α-CA	L + Z	LY	β-CR
Australia $N = 51$ Canfield et al. (2003)	Total volume from one breast between 13:00 and 17:00; electric pump	HPLC µmol/L	0.060 ± 0.007	0.034 ± 0.003	0.027 ± 0.002	0.031 ± 0.002	0.024 ± 0.002
Canada $N = 55$ Canfield et al. (2003)			0.036 ± 0.003	0.036 ± 0.003	0.030 ± 0.001	0.030 ± 0.002	0.027 ± 0.003
Chile $N = 49$ Canfield et al. (2003)			0.044 ± 0.004	0.024 ± 0.002	0.057 ± 0.005	0.021 ± 0.002	0.016 ± 0.002
Japan $N = 50$ Canfield et al. (2003)			0.062 ± 0.005	0.045 ± 0.004	0.077 ± 0.002	0.023 ± 0.002	0.080 ± 0.008
Mexico $N = 50$ Canfield et al. (2003)			0.051 ± 0.005	0.031 ± 0.002	0.044 ± 0.003	0.032 ± 0.002	0.057 ± 0.008
Philippines $N = 60$ Canfield et al. (2003)			0.022 ± 0.002	0.041 ± 0.010	0.035 ± 0.003	0.016 ± 0.002	0.012 ± 0.001
UK $N = 50$ Canfield et al. (2003)			0.048 ± 0.003	0.031 ± 0.003	0.027 ± 0.002	0.034 ± 0.002	0.012 ± 0.001
Brazil $N = 49$ Meneses and Trugo (2005)	Total volume from one breast; handheld breast pump	HPLC µmol/L	0.018 ± 0.002	–	0.006 ± 0.001	–	–
Korea $N = 34$ Duan et al. (2019)	Lack of data	HPLC µg/100 g	1.68 (0.49–9.46)	0.19 (0.03–1.07)	3.85 (1.15–9.68)	0.29 (0.00–1.22)	3.60 (0.53–10.96)
China $N = 56$ Xu et al. (2019)	Total volume from one breast between 13:00 and 16:00; manual pump	HPLC µg/dL	–	–	L: 8.2 ± 0.65 *trans*-L: 7.7 ± 0.61 Z: 2.0 ± 0.17	–	–

Korea $N = 98$ Kim et al. (2018)	150 ml; breast pump	HPLC μg/dL	–	–	3.50 ± 3.71	–	–
Poland $N = 53$ Zielinska et al. (2019)	Pooled sample from 4 samples collected 4 times during the day (6:00–12:00; 12:00–18:00; 18:00–24:00; 24:00–6:00) via collection of the same amount of pre- and postfed milk; manually or breast pump	HPLC nmol/L	33.1 (32.9–33.3)	–	33.0 (24.1–41.8)	111.2 (105.0–117.3)	–
China $N = 111$ Nguen et al. (2020)	50–150 ml (lack of other data); manually or breast pump	HPLC μgl/L	–	–	66.1 ± 51.6	–	–
Korea $N = 155$ Nguen et al. (2020)			–	–	41.3 ± 27.4		
Pakistan $N = 97$ Nguen et al. (2020)			–	–	47.6 ± 58.9	–	–
Vietnam $N = 92$ Nguen et al. (2020)			–	–	49.7 ± 47.9	–	–
Cambodia $N = 23$ Whitefield et al. (2020)	Total volume from one breast; electric pump	HPLC μgl/L	63.4 (41.4–97.1)	11.4 (8.59–15.2)	56.1 (40.7–77.3)	8.87 (7.22–10.9)	10.2 (7.36–13.9)
Indonesia $N = 212$ Gibson et al. (2020)	Total volume from one breast at morning; handheld breast pump	HPLC μgl/L	21 (14–32)	9.6 (6.2–12.6)	–	–	26 (17–43)

α-CA = α-carotene. β-CA = β-carotene, β-CR = β-cryptoxanthin. HPLC = high-performance liquid chromatography. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. Z = zeaxanthin

Table 3 Carotenoid intake during lactation

Study group	Nutritional assessment method	Dietary supplements assessed	Nutritional database used	Assessed carotenoids, units	Carotenoid dietary intake	Associations to breastmilk carotenoids
Multinational study Canfield et al. (2003)	24 h food record	No	No	Rank order of intake	No quantitative data; qualitative analysis of dietary sources and rank order of carotenoids according to its intake	Rank order in breastmilk and dietary carotenoids was similar and suggests an association
Brazil $N = 49$ Meneses and Trugo (2005)	FFQ	No	ESHA database	Provitamin A carotenoids, μg RAE/d	301 ± 184	NS
Italy $N = 21$ Cena et al. (2009)	2 × quantitative 30-item FFQ covering past month (3- and 30-day T0; T1)	No	No data	L, μg/d	T0 L: 1209 ± 157 T1 L: 1258 ± 102	T0: $r = 0.94$, $p \leq 0.001$ T1: $r = 0.82$, $p \leq 0.001$
USA $N = 21$ Panagos et al. (2016)	3-day food record 1 week before visit	No	USDA	β-CA, α-CA, LY, L + Z, CR, mg/ 1000 kcal	β-CA: 2.35 ± 3.19 α-CA: 0.31 ± 0.35 LY: 1.63 ± 1.55 L + Z: 2.08 ± 2.92 CR: 0.11 ± 0.15	L: $\beta = 0.41$, $p < 0.01$
China $N = 509$ Xue et al. (2017)	1 × 24 h dietary record	Yes	Chinese and Japanese food composition tables	Total carotenoids	No data	NS

Korea $N = 98$ Kim et al. (2018)	3-day food record	Yes	USDA	L, β-CA mg/day	β-CA: 4.33 ± 2.61 L: 4.71 ± 3.11	$\beta = 0.3653$, $p = 0.0022$
China $N = 56$ Xu et al. (2019)	1 × 3-day food record	No	USDA	L + Z, mg/d	3.3 ± 0.41	NS
Poland $N = 53$ Zielinska et al. (2019)	2 × 3-day food record (3- and 6-month T1;T2)	Yes	USDA	β-CA, LY, L + Z, μg/d	T1: β-CA: 4480.8 (3575.0–5386.7) LY: 7898.3 (5465.2–10329.5) L + Z: 2945.2 (1910.8–3979.6) T2: β-CA: 3441.9 (5037.5–9474.1) LY: 7255.8 (5037.5–9474.1) L + Z: 3739.3 (2834.9–4643.7)	T1: β-CA: $\beta = 0.407$, $p \leq 0.01$ LY: $\beta = 0.415$, $p \leq 0.01$ L + Z: $\beta = 0.730$, $p \leq 0.001$ T2: β-CA: $\beta = 0.428$, $p \leq 0.001$ LY: $\beta = 0.401$, $p \leq 0.01$ L + Z: $\beta = 0.644$, $p \leq 0.001$
Brazil $N = 19$ Machado et al. (2019)	2 × 24 h dietary record	No	USDA	β-CA, α-CA, LY, L + Z, μg/d	β-CA: 3249 (1408–6707) α-CA: 1053 (56–3712) LY: 1854 (302–6472) L + Z: 2446 (872–4873)	β-CA: $\beta = 2.52 \times 10^{-5}$, $p \leq 0.05$
Serbia $N = 19$ Denić et al. (2019)	1 × 7-item FFQ covering pregnancy and lactation	No	–	–	–	β-CA correlated to vegetable and fruit intake

α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene. β-CR = β-cryptoxanthin. FFQ = food frequency questionnaire. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. RAE = retinol activity equivalent. USDA = United States Department of Agriculture. Z = zeaxanthin

intervention studies confirmed a strong association between dietary intake of carotenoids and their breastmilk levels (Sherry et al. 2014; Nagayama et al. 2014; Haftel et al. 2015; Schaefer et al. 2020).

Assays for Carotenoids: Methodological Implications

Breastmilk Sample Collection

Studies assessing breastmilk carotenoids used a variety of breastmilk collection methods, sometimes not even described properly (Tables 1 and 2). Differences in milk sampling protocol may make it difficult to compare the results and explain discrepancies in carotenoid level between studies (Zielinska et al. 2017a; Denić et al. 2019). To minimize the bias regarding sampling, the selecting of optimal and appropriate milk collection method (and its description) is crucial in the case of breastmilk carotenoid assessment (Miller et al. 2013; Zielinska et al. 2017a). There is no recommended, universal method of breastmilk sampling for milk composition studies (Miller et al. 2013). Milk collection protocol adapted for studies regarding carotenoids (and other fat-soluble compounds) should be standardized among all study subjects and several conditions considered, including longitudinal, circadian, and within-feeding variations in milk composition, the volume of milk consumed at the previous feeding, intervals between feedings, expression method (e.g., availability of breast pumps), physiological milk let-down, and infant's nutritional needs (Miller et al. 2013; Samuel et al. 2020; Casavale et al. 2019). Miller et al. (2013) characterized the most often used milk sampling protocols: full breast expression, expression of the alternate breast, mid-feed sampling, fore- and hindmilk sampling, and foremilk (or hindmilk) sampling. In studies assessing carotenoids, full breast expression was used most often (Schweigert et al. 2004; Song et al. 2013; Lipkie et al. 2015; Xue et al. 2017; Ríos et al. 2017; Xavier et al. 2018; Redeuil et al. 2021; Wu et al. 2021; Canfield et al. 2003; Meneses and Trugo 2005; Whitefield et al. 2020; Gibson et al. 2020). Several studies used fore- or hindmilk sampling (Macias and Schweigert 2001; Cena et al. 2009; Machado et al. 2019; Nguen et al. 2020) and one study fore- and hindmilk sampling four times per day (Zielinska et al. 2019a). Full breast expression is a relative optimal method that eliminates the within-feeding variation in milk composition but does not consider circadian variations, so the time of sampling should be specified in one study group (Miller et al. 2013). Fore- or hindmilk sampling does consider neither circadian variation nor within-feeding variation, so it is less applicable (Miller et al. 2013). Fore- and hindmilk sampling eliminates bias related to within-feeding variations in milk composition and requires a relatively small amount of milk, so it is not related with the nutritional risk for infant (Miller et al. 2013). Repeatable collections within the 24-h period will minimize bias related to circadian variation (Zielinska et al. 2019a), so this method is more applicable than the previous one. Regardless of the chosen sampling protocol method of milk expression, data about breast from the sample

was expressed, the volume of collected sample and infant feeding data should be also specified in further studies (Samuel et al. 2020).

Breastmilk Sample Handling and Storage

Handling of collected breastmilk samples is another critical step in milk analysis (Miller et al. 2013; Samuel et al. 2020). Carotenoids are susceptible to oxygen, light, temperature, and prooxidant metal, so invalid sample handling and storage may lead to carotenoid loss due to its autooxidation or *cis-trans* isomerization (Jackson et al. 1998; Canfield et al. 2003; Rodríguez-Bernaldo de Quirós and Costa, 2006; Amorim-Carrilho et al. 2014). Breastmilk samples should be expressed immediately into the collection container, protected against oxygen and light, and kept in the cooling condition during transport to the laboratory (Samuel et al. 2020). Before preparing aliquots for further analysis breastmilk should be homogenized with gentle swirls due to the even distribution of fat into samples (Miller et al. 2013). The milk sample should be divided into smaller aliquots and then stored as soon as possible at −80 °C until further analysis. A previous study showed that 1 year of storage in −70 °C resulted in the decrease of β-cryptoxanthin, but other carotenoids were stable (Jackson et al. 1998). It is important to avoid freeze-thaw cycles that lead to lipolysis activation. Before analysis stored samples should be thawed at room temperature or in a water bath (37 °C) (Samuel et al. 2020).

Breastmilk Analysis

Analysis of breastmilk carotenoids covers several stages (Fig. 2). The most popular method of breastmilk carotenoids analysis is a normal/reversed-phase HPLC/UHPLC analysis which requires a specific sample preparation (Table 4). The applied sample preparation methods vary depending on the analytical material and chemical form of carotenoids. In plants tissues, except bell pepper fruits, carotenoids abundant mostly in free form. Animals tissue, including breastmilk, contain mostly fat-esterified carotenoids form. From chemical point of view they are more stabile, but for analysis purposes saponification process should be used. For the extraction of free carotenoids solvents such as hexane or acetone are sufficient, but for breastmilk samples prior to extraction saponification and hydrolysis of the ester bonds have to be conducted (Amorim-Carrilho et al. 2014) with a strong base solution. For the saponification of breastmilk samples, the most commonly used reagent is potassium hydroxide (30–60%) in methanol and for hydrolysis – ethanol. However, saponification may result in the loss of transformation of carotenoids, so the optimal parameters of temperature and time are crucial (Jackson et al. 1998; Amorim-Carrilho et al. 2014). For the prevention of carotenoid loss during sample preparations, a variety of reagents were added, whereas the most common were butylated-hydroxytoluene (BHT) and pyrogallol (Table 4; Amorim-Carrilho et al. 2014). After saponification and hydrolysis, breastmilk carotenoids have to be extracted from

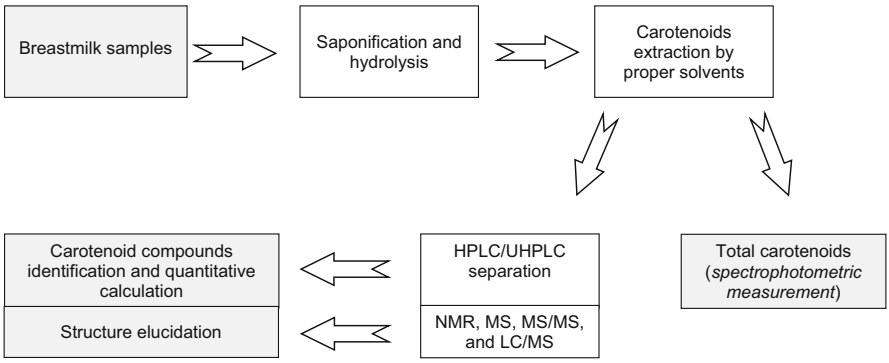

Fig. 2 Scheme of sample preparation and carotenoid analysis. HPLC = high-performance liquid chromatography, LC/MS = liquid chromatography-mass spectrometry, MS = mass spectrometry, MS/MS = tandem mass spectrometry, NMR = nuclear magnetic resonance spectroscopy, UHPLC = ultra-high-performance liquid chromatography

breastmilk sample, usually by n-hexane in 1–3 repeatable cycles (Table 4; Amorim-Carrilho et al. 2014). After those procedures the collected extract of breastmilk sample is purified from the extraction solvent by evaporating under pressure and re-dissolving the sample in the final solvent (usually hexane or mobile phase). The sample prepared in this way can be used for the measurement of total carotenoids (in the spectrophotometric method) or for the separation and qualitative and quantitative identification in the HPLC/UHPLC system. Based on retention time and pure carotenoid standards prepared, the sample will be identified quantitatively and/or qualitatively.

Carotenoids absorb light very strongly and have intense absorption ranges in the visible light regime. The features of the UV/Vis spectrum present the characteristic information and these can be use for qualitative and quantitative analysis of carotenoids. The first and most important feature is the wavelength. For carotenoids, it is 445–475 nm (Miller et al. 2013). The second feature is the shape and arrangement of the spectrum, and the third is the absorbance value. All this information can be obtained graphically (spectrum illustration) and presented numerically (measurable value). The type of solvent used for the mobile phase should be taken into account when determining carotenoids in breastmilk samples. For practical reasons, light absorption is best for most carotenoids; they are similar in hexane, diethyl ether, and acetonitrile. Other solvents absorb light, so there are bathochromic shifts. The presence of water is undesirable. This can also affect light absorption. The expected concentration of carotenoids in the examined sample is also of great importance (Amorim-Carrilho et al. 2014).

In general, carotenoids are particularly difficult to analyze accurately due to their lipophilicity, instability, structural similarity, and/or scarcity of certified reference materials, which poses problems of accuracy and comparability of obtained results.

Table 4 Analytical methods for carotenoid assessment in breastmilk samples

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
USA $N = 23$ Jackson et al. (1998)	β-CA, α-CA, L + Z, LY, β-CR	–	Saponification: 2 mL of mixed milk +1.5 ml of 50% (wt./v) KOH + 2.5 ml ethanol. Water bath (2 h, 45 °C). 3x extraction: 3 ml hexane +0.025% (wt./v) BHT. Evaporation of hexane residue under nitrogen, then dissolved in mobile phase	HPLC RP (reversed phase) UV-Vis detectors Column: C18 (3.9 × 300 mm, 4 μm) No guard column Mobile phase: isocratic acetonitrile/methanol/THF (50:45:5; (v/v/v) Injection: 200 μl Flow rate: 2.5 ml/min Time: 20 min
Cuba $N = 21$ Macias and Schweigert (2001)	β-CA, α-CA, L, Z, LY, β-CR	–	Saponification: 1 ml milk +500 μl 12% pyrogallol in ethanol+1.5 ml 50% KOH and ethanol (3:5; v/v), vortex, incubation (2 h, 37 °C, under nitrogen). 2x extraction: +1 ml n-hexane (10 min, centrifugation); +1.5 ml 0.1 M NaCl +500 μl ethanol, vortex (centrifugation). Evaporation of hexane residue under nitrogen, then dissolved in 200 μl isopropanol, vortexed and sonicated	HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) Mobile phase: A (methanol/methyl-tert-butyl-ether/water, 83:15:2; v/v/v); B (methanol/methyl-tert-butyl-ether/water, 8:90:2; v/v/v) both with 1.5% ammonium acetate in H_2O Gradient elution Flow rate: 1 ml/min
Germany $N = 21$ Schweigert et al. (2004), Schweigert et al. (2000)	β-CA, α-CA, L, Z, LY, β-CR, 9-cis-β-CA, canthaxanthin	–		HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) and C18 pre-column Mobile phase: A (methanol/water, (90:10; v/v), with 0.4 g ammonium acetate in 1 l H_2O); B (methanol/methyl-tert-butyl-ether/water (8:90:2; v/v/v), with 0.1 g ammonium acetate in 1 l H_2O)

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
Multinational study Canfield et al. (2003), Liu et al. (1998)	β-CA, α-CA, L + Z, LY, β-CR	Creamatocrit	Hydrolysis and saponification: 1 ml milk +10 mg $MgCO_3$ + 6 mg bile salt. Incubation in an orbital shaker (1 h); + 1 mg or protease +10 mg of lipase; incubation (1 h); + 1 ml KOH:H_2O (1:1; wt./v); incubation (0.5 h, 37 °C). Extraction: +0.5 ml of ethanol (vortexing, 30 s, room temperature/10 min); + 2 ml hexane; vortexing (1 min); evaporation under nitrogen, then dissolved in 250 µl o THF/CAN (15:85; v/v); centrifuging (12,700x*g*, 15 s.)	HPLC UV-Vis detectors Column: C18 (4.6 × 250 mm, 5 µm) Mobile phase: 95%A (ACN:THF (85:15; v/v); 5% B (50 mM ammonium acetate in methanol with 0.05% TEA) Flow rate: 2.5 ml/min Time: 13 min
Brazil $N = 49$ Meneses and Trugo (2005)	β-CA, L + Z	Creamatocrit		HPLC UV-VIS Column: C18 (4.6 × 250 mm, 5 µm) Mobile phase: 95% A (ACN:THF, 85:15; v/v), 5% B (50 mmol/L ammonium acetate in methanol) Injection: 20 µl Flow rate: 2 ml/min
Brazil $N = 19$ Machado et al. (2019)	β-CA, α-CA, L + Z, LY	Creamatocrit		HPLC UV-Vis detectors Column: C18 (4.6 × 150 mm) Mobile phase: ACN/THF+ 15 mM methanolic ammonium acetate, 65:25:10, v/v/v) Injection: 50 µl Flow rate: 0.9 ml/min
Italy $N = 21$ Cena et al. (2009)	L	–	Saponification: 2 ml milk +0.5 ml of KOH (40% in methanol) + 0.1 ml β-apo-8′-carotenal in methanol, incubation in water bath (30 min, 45 °C). 3 x extraction: + 1.5 ml hexane +0.01% BHT wt./v; evaporation under nitrogen, then dissolved in 0.5 ml of isopropanol-hexane (10: 90; v/v)	Column: C18 (4 × 250 mm, 4 µm) Mobile phase: mixture of A (2-propanol), B (hexane) Gradient elution Flow rate: 1 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
USA $N = 17$ Song et al. (2013)	β-CA, α-CA, L, Z, LY, β-CR; α-CR	Creamatocrit	Saponification: 0.75 ml + 0.3 ml 30% methanolic KOH (15 min, ambient temperature); 3 x extraction: 3:1 petroleum ether +0.1% 2,6-di-tert-butyl-4-methylphenol: acetone; evaporation under vacuum, then dissolved in 150 µl of 1:1 ethyl acetate and methanol before analysis	HPLC DAD detectors Column: C30 (2 × 150 mm) + guard column (2 x 50 mm)
USA $N = 20$ Lipkie et al. (2015)	β-CA, α-CA, L, Z, LY, β-CR, α-CR	Creamatocrit	Saponification: 0.7 ml milk +1.3 ml 0.9% NaCl +2 ml ethanol + internal standard; shaken 10 min. 2 x extraction: + 2 ml (9:1; v/v) hexane/ethyl acetate +0.1% BHT. Evaporation under nitrogen at 35 °C, then placed on ice. Saponification: + 2 ml 10% wt./v KOH, incubation (1 h, 37 °C); quenched with 2 ml chilled water; re-extraction 9:1 ethanolic extract, dried, resolubilized in 50 µl ethyl acetate +50 µl methanol; centrifugation (14,000 rpm/5 min)	HPLC DAD detectors Column: C30 (2 × 150 mm) Mobile phase: mixture of 2 mM ammonium acetate and ethyl acetate
USA $N = 21$ Panagos et al. (2016)	β-CA, LY, L, Z, β-CR	Ultrasound technique	Saponification: 4 ml milk +5 ml ethanol +3 ml 50% wt./v) KOH; sonication in a water bath, saponification in an orbital shaker (25 °C, 130 oscillations/min: β-CA, L, Z, CR: 16 h, α-CA, LY: 0.5 h); 2 x extraction +4 ml hexane, vortexing and sonicating (5 min); evaporation under nitrogen, then dissolved in 2.5 ml ethanol and 3.5 ml of	HPLC RP (reversed phase) Column: C30 (150 × 4.6 mm, 3 µm) Mobile phase: 10% THF, 90% methanol (9:1; v/v), and 0.5 g/l BHT Flow rate: 1.6 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
			H_2O, centrifugation 600x*g*/10 min; hexane layers evaporated in nitrogen and dissolved in 200 µl of THF methanol (20:80, v/v)	
China *N* = 509 Xue et al. (2017)	β-CA, α-CA, LY, L, Z, CR	–	Saponification: 1 ml milk +5 µl ethanol + BHT (79 g/l) + 10 µl aqueous solution of deferoxamine mesylate (10 mg/ml) + 4 ml methanol +1 ml KOH (30%, v/v), mixing, water bath (30 min, 37 °C); cooling on ice; 2x extracting: + 5 ml hexane +350 mg BHT/l, mixing, 30 s, centrifugation (2500 rpm/10 min, 4 °C); hexane layers evaporated in nitrogen and dissolved in 70 µl dioxane/ethanol (1:1; v/v) + 70 µl acetonitrile; centrifuging 2500 rpm / 10 min	UHPLC UV-Vis detectors Column: C18 (2.1 mm × 150 mm, 1.8 µm) Mobile phase: A (0.5 M ammonium acetate in water) Flow rate: 0.4 ml/min
Spain *N* = 30 Ríos et al. (2017)	β-CA, α-CA, L, Z, LY, β-CR; xanthophyll esters	Solvent extraction followed by gravimetry	2 x extraction: 3 ml milk +6 ml methanol, vortex 20 min, cooled −20 °C, centrifugation (10,000 x *g*, 5 min, 4 °C); upper layer was discarded +5 ml diethyl ether +2 ml hexane, vortexing, centrifuging (10,000 x *g*, 5 min, 4 °C); evaporation on rotatory evaporator (25 °C), dissolved in 1 ml hexane; SPE column, elution with 30 ml hexane; evaporation on rotary evaporator (25 °C), dilution in 1 ml methanol/methyl-tert-butyl-ether (8:2; v/v)	HPLC-MS Column: C30 (250 × 4.6 mm, 3 µm) Mobile phase: methyl-tert-butyl-ether/water (A: 81:15:14; B: 7:90:3) Gradient elution Injection: 30 µl Flow rate: 1 ml/min
Spain *N* = 70 Xavier et al. (2018)	α + β-CA, L, Z, LY, β-CR; xanthophyll esters			UHPLC

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
China $N = 56$ Xu et al. (2019)	L, Z, trans-L	–	Milk sample + tetrahydrofuran; Saponification: + 5% methanolic KOH; extraction: 50 mg BHT +200 ml dichloromethane +400 ml petro ether +400 ml hexane	HPLC PAD detectors Column: C30 (150 × 4.6 mm, 3 μm) Mobile phase: methyl-tert-butyl-ether Gradient elution Injection: 50 μl Flow rate: 1 ml/min
Korea $N = 98$ Kim et al. (2018)	L	–	1 ml milk +8 ml acetone +0.1% BHT; incubation (20 min, 37 °C); centrifugation (3000 rpm, 10 min); filtrating through a syringe filter	HPLC RP (reversed phase) Column: C30 (250 × 4.6 mm, 5 μm) Mobile phase: methanol/methyl-tert-butyl-ether Gradient elution Flow rate: 1 ml/min
Korea $N = 34$ Duan et al. (2019)	β-CA, α-CA, LY, L + Z, β-CR	MilkoScan FT2	5 g of milk +10 ml pyrogallol solution (6% in ethanol); vortex (2 min), evaporation with nitrogen (1 min); sonication (10 min) + 8 ml KOH (60%); vortex (2 min), water bath 60 min, 75 °C, 100 rpm) + 30 ml 3% NaCl +15 ml hexane/ethyl acetate (85:15; v/v, 0,01 BHT), vortex	HPLC UV-Vis detectors Column: C18 (250 × 4.6 mm, 5 μm) Mobile phase: acetonitrile/methanol/methylene, (75:20:5, v/v/v) Gradient elution Injection: 20 μl Flow rate: 1 ml/min Time: 40 min
Poland $N = 53$ Zielinska et al. (2019)	β-CA, LY, L + Z	MIRIS HMA	Saponification: 2 ml milk +500 μl 12% pyrogallol +50 μl 1% ascorbic acid in 0.1 M HCL, + 1.5 ml 30% KOH + 2.5 ml ethanol, vortexing (30 sec), incubation in water bath (40 min, 50 °C); ice cooling; 3 x extraction: + 1 ml NaCl +1 ml n-hexane; shaking 3 min; centrifugation (8000 rpm, 10 min, 4 °C); evaporation in vacuum (30 min, 30 °C); dissolved in 0.5 ml hexane	HPLC UV-Vis detectors Column: C18 (250 × 4.6 mm) Mobile phase: A (acetonitrile/methanol, 90:10, v/v) and B (methanol/ethyl acetate (34:16, v/v) Injection: 100 μl Flow rate: 1 ml/min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
Multinational study Nguen et al. (2020)	L	–	Saponification: 2 ml milk +4 ml ethanol (0.1% wt./v) + 1 ml NaCl (2% wt./v) + 4% ascorbic acid +1 ml KOH (60%, wt./v), incubation (70 °C, 60 min), cooling in ice water. 2 x extraction: 5 ml hexane, + 0.1% NaCl; evaporation under vacuum (40 °C, 30 min), dissolved in 100 µl isopropanol/hexane (75: 25 v/v; 0.025% BHT)	HPLC UV-Vis detectors Column: C30 (150 × 4.6 mm, 5 µm) Mobile phase: A (methanol/ acetonitrile/water, 4: 5:1; v/v/v) B (methyl-tert-butyl) Gradient elution Flow rate: 1 ml/min
Cambodia $N = 23$ Whitefield et al. (2020), Turner and Burri (2012)	β-CA, α-CA, LY, L + Z, β-CR	SpectraStar 2600 XT Neonatal Analyzer	Saponification: 100 µl of milk +0.1% BHT in ethanol +10% pyrogallol in ethanol +200 µl 15% KOH. Extraction: + 4 ml hexane, evaporation, dissolved in 50 µl acetonitrile	HPLC UV-Vis detectors Column: C18 (100 × 2.1 mm, 3.5 µm) Injection: 20 µl
Bangladesh $N = 18$ Hampel et al. (2017)	β-CA	Creamatocrit		HPLC UV-Vis detectors Column: C18 (100 × 2.1 mm, 3.5 µm) Injection: 25 µl Flow rate: 0.6 ml/min
Indonesia $N = 212$ Gibson et al. (2020)	β-CA, α-CA, β-CR	Creamatocrit		
Switzerland $N = 28$ Redeuil et al. (2021), Levêques et al. (2019)	β-CA, L, Z, LY, β-CR	–	750 µl milk +5 µl BHT ethanolic solution (79 g/ l) + 10 µl deferoxamine mesylate aqueous solution (10 mg/ml) + 1 ml ethanol +25 µl internal standard, mixing; 2 x extraction: + 2.5 ml n-hexane-ethyl acetate (90:10; v/v) with 350 mg/l BHT, mixing (4 min), centrifugation (1200 x g, 10 min, 4 °C); + n-hexane-ethyl acetate (90: 10; v/v), evaporation and dissolved in 125 µl isooctane-ethyl acetate (90:10; v/v), centrifugation (11,000 x *g*, 10 min, room temperature)	HPLC UV-Vis detectors Mobile phase: A (n-hexane) B (n-hexane-dioxane, 50:50, v/v + 0.001% acetic acid) Gradient elution Injection: 5 µl Flow rate: 0.3–0.4 ml/min Time: 22 min

(continued)

Table 4 (continued)

Study	Analyte	Analytical method		
		Milk fat analysis	Extraction protocol	Detection system
China $N = 42$ Wu et al. (2020), Schimpf et al. (2017)	β-CA, L, Z, LY, β-CR	–	0.5 ml milk +4 ml water +0.5 g sodium ascorbate, 10 ml methanol + 1 ml tetrahydrofuran. Saponification: 1 ml aqueous KOH (45%, wt./v), water bath (65 °C, 15 min); extraction: +10 ml hexane-methyl-tert-butyl-ether (3:1; v/v); evaporation and dissolved in 400 µl hexane-methyl-tert-butyl-ether	HPLC UV-Vis detectors Column: C30 (250 x 4.6 mm, 3 µm) Mobile phase: A (methanol) B (hexane-methyl-tert-butyl-ether) Gradient elution Injection: 5 µl Flow rate: 0.3–0.4 ml/min Time: 22 min

ACN = acetonitrile. BHT = butylatedhydroxytoluene. α-CA = α-carotene. α-CR = α-cryptoxanthin. β-CA = β-carotene. β-CR = β-cryptoxanthin. HCl = hydrochloric acid. HPLC = high-performance liquid chromatography. KOH = potassium hydroxide. L = lutein. LY = lycopene. L + Z = lutein+zeaxanthin. UHPLC = ultra-high-performance liquid chromatography. HPLC-MS = high-performance liquid chromatography-mass spectrometry. THF = tetrahydrofuran. UPLC = ultra-performance liquid chromatography. Z = zeaxanthin

Nutritional Assessment

Data about maternal nutrition was collected using a variety of methods (Table 3): FFQ covering different periods (Meneses and Trugo 2005; Cena et al. 2009; Denić et al. 2019), 24-food record (Canfield et al. 2003; Xue et al. 2017; Machado et al. 2019), and 3-day dietary record (Panagos et al. 2016; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a) repeated one (Canfield et al. 2003; Meneses and Trugo 2005; Panagos et al. 2016; Xue et al. 2017, Kim et al. 2018; Xu et al. 2019; Denić et al. 2019) or more times. Studies evaluated the association between breastmilk carotenoids and the intake of specific food groups (Canfield et al. 2003; Denić et al. 2019) or the intake of total carotenoids (Meneses and Trugo 2005) or specific carotenoid (Cena et al. 2009; Xue et al. 2017; Panagos et al. 2016; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a; Machado et al. 2019). Studies assessing carotenoid intake mainly calculated it based on USDA food database because specific national food databases are often lacking in data about carotenoids in different foods (Amorim-Carrilho et al. 2014; Panagos et al. 2016; Xue et al. 2017; Kim et al. 2018; Xu et al. 2019; Zielinska et al. 2019a; Machado et al. 2019). Unfortunately, data collection in a short period of time, lack of national database of carotenoid contents in foods, or collection of data via FFQ may lead to inaccurate evaluation of carotenoid intake (Amorim-Carrilho et al. 2014). In consequence, this may lead to a lack of bias in the analysis of association between maternal intake of carotenoids and its breastmilk concentrations.

Data Collection

The most recommended method of the collection of dietary data is at least a 3-day dietary record, which allows to collect all abundant information about consumed foods, its portion size, and its preparation method, as well as minimize the bias related to day-to-day variation, allowing the calculation of energy and nutrient intake (Shim et al. 2014; Ortega et al. 2015; Burrows et al. 2017). Carotenoid's bioavailability increases in the presence of dietary fat and after selected food processing (e.g., chopping and cooking), but reduces in the presence of dietary fiber (Reboul 2019; Böhm et al. 2021), especially soluble fractions (pectin) (Hamułka 2009). Also, individual genetic characteristics related to intestinal transporters and digestion enzymes may influence its bioavailability (Krinsky and Johnson 2005; Böhm et al. 2021). The 3–7-day records allow obtaining all the nutritional data that influence carotenoid bioavailability. However, this method is related to higher costs and time and it is difficult for participants; when the use of the 3–7-day dietary record is impossible, then the use of semiquantitative FFQ may be useful (Shim et al. 2014). Another possibility is a qualitative analysis of maternal nutrition. Previous study confirmed correlations between FFQ and plasma carotenoids in plasma. Moreover, FFQ covers a longer period of time, so it applies to many types of vegetables and fruits consumed seasonally (Burrows et al. 2017; Yuan et al. 2018). Previously it was shown that fruit and vegetable intake assessed by FFQ was correlated with plasma carotenoids (Burrows et al. 2015), which are a good biomarker of their intake (Baldrick et al. 2011). However, the use of FFQ should be validated and it should include not only different types of vegetables and fruits but also different methods of their preparation as these can affect carotenoid bioavailability (Burrows et al. 2015; Dias et al. 2018). The utilization of semiquantitative or qualitative FFQ is especially useful when the study has a cross-sectional design and assessed the maternal nutrition and biomarkers (Burrows et al. 2017). In the studies conducted, biomarkers may not reflect the same period as in nutritional assessment, e.g., 24-food record, so using both methods of collection of nutritional data may be beneficial. Another important issue, which should be considered during nutritional assessment, is the usage of dietary supplements, which is very common among pregnant and lactating individuals. As many supplements contain carotenoids (Böhm et al. 2021), assessment should include also quantitative evaluation of carotenoid intake via dietary supplements.

Carotenoid Database

A proper assessment of semiquantitative and quantitative data requires a valid database of carotenoid level and isoforms in nationally available food products, including different crop varieties (Amorim-Carrilho et al. 2014; Dias et al. 2021). So, the development those databases covering different food products and methods of preparation is a crucial step in the studies assessing carotenoid intake, because carotenoid levels are different in raw and processed products (Dias et al. 2018).

Moreover, it is important to include less common carotenoids, such as astaxanthin, phytoene, phytofluene, capsanthin, canthaxanthin, and fucoxanthin (Böhm et al. 2021; Liu et al. 2021).

Considering that foods contain different carotenoids in different levels, it is recommended to consume a diversified diet to obtain appropriate levels of the major health-promoting dietary carotenoids.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, we reviewed studies and methods assessing breastmilk carotenoids and their maternal intake. Previous studies showed that breastmilk carotenoids are highly correlated with infant carotenoid status (Henriksen et al. 2013; Sherry et al. 2014; Lipkie et al. 2015; Sun et al. 2021). Moreover, before complementary feeding starts breastmilk is the only source of carotenoids for infants, as the infant formula is not supplemented with carotenoids (Jewell et al. 2004), and breastfed infants have higher plasma carotenoid levels than those formula fed (Sommerburg et al. 2000; Bettler et al. 2010). Interestingly, breastfeeding may have a longitudinal impact on children's carotenoid status in childhood (Liu et al. 2021). Currently, the potential health benefits of carotenoids in early postnatal development were mostly investigated for lutein and zeaxanthin (Giordano and Quadro 2018). Carotenoids are very effective antioxidants and immunomodulators (Krinsky and Johnson 2005; Jomova and Valko 2013; Zielinska et al. 2017a, b; Giordano and Quadro 2018). Xanthophylls, lutein, zeaxanthin, and β-cryptoxanthin are accumulated in retina and brain regions related to cognitive function and probably are crucial for optimal neurodevelopment and visual performance (Henriksen et al. 2013; Jeon et al. 2018). Breastmilk and maternal lutein were associated with a better recognition memory in infancy (Cheatham and Sheppard 2015) and visual acuity (Lai et al. 2020). In other studies, breastmilk β-carotene was related to infant motor development at the 6 months of life (Zielinska et al. 2019b). It was hypothesized that lutein and zeaxanthin may protect premature infants against elevated oxidative stress and diseases related to prematurity, such as retinopathy of prematurity but results are inconclusive and further research is needed (Cota et al. 2020). There is no doubt that higher intake of carotenoids, especially with vegetables and fruits, will be also associated to health benefits in mothers, including reduced risk of noncommunicable diseases and maintenance of cognitive performance (Fig. 3) (Krinsky and Johnson 2005; Johnson 2014; Zielińska et al. 2017b; Giordano and Quadro 2018).

Mini-Dictionary of Terms

- ***Breastmilk***. *Complex and dynamic biological fluid rich in nutrients and bioactive compounds produced in mammary glands during lactogenesis.*
- ***Carotenes.*** *Non-oxygenated, less polar, the fraction of carotenoids,* e.g. *β-carotene, α-carotene, lycopene.*

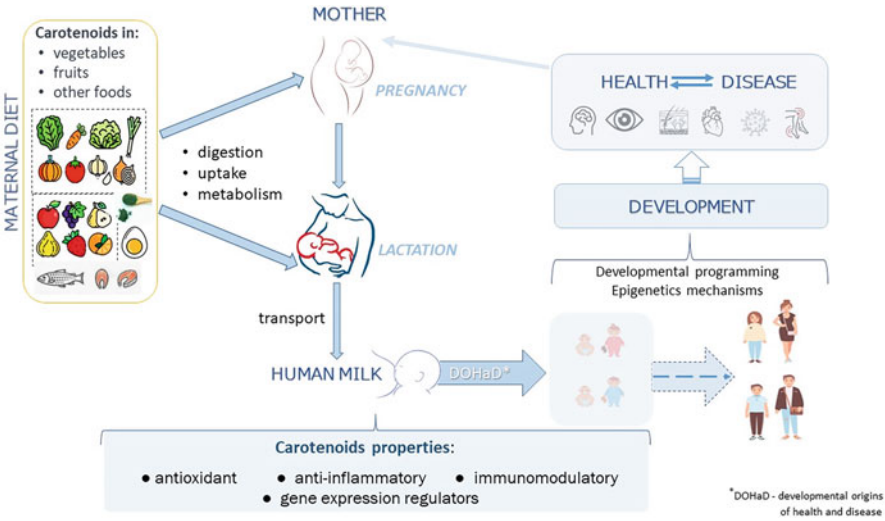

Fig. 3 Association between maternal carotenoid intake, breastmilk, and children development and health. DOHaD = Developmental Origin of Health and Disease. Skin by Mahmure Alp from the Noun Project

- ***Carotenoids.*** *Fat-soluble, natural pigments produced by plants and microorganisms and are responsible for the color of a variety of foods. Some carotenoids have provitamin A properties (β-carotene, α-carotene,* β-cryptoxanthin).
- ***Colostrum.*** *Breastmilk is produced in low volume after birth: low in fat, high in protein, immune factors. Thanks to high levels of carotenoids, colostrum is often yellow or orange in color.*
- ***Foremilk.*** *The first milk at the beginning of every single feeding. Compared to hindmilk it contains a little amount of fat (1–2%).*
- ***Hindmilk.*** *Breastmilk at the end of every single feeding. Compared to foremilk it contains more fat and carotenoids.*
- ***Mature milk.*** *Breastmilk produced since 3–4 weeks postpartum*
- ***Transitional milk.*** *Breastmilk produced after colostrum and mature milk. The fat concentrations, energy value, and volume gradually increase in transitional milk.*
- **Xanthophylls.** Oxygenated hydrocarbon molecules, more polar than carotenes, e.g., lutein and zeaxanthin (L + Z), β-cryptoxanthin, and astaxanthin.

Key Facts of Carotenoids in the Breastmilk

- Humans cannot synthesize carotenoids and rely on their carotenoid intake with diet, mainly vegetables and fruits, algae, or some animal foods, e.g., egg yolk, salmon, and rainbow trout.
- Major dietary, plasma, and breastmilk carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene, and β-cryptoxanthin.

- Breastmilk carotenoids are related to maternal dietary intake and influence infant carotenoid status.
- Dietary carotenoids may influence infant growth and development and health in later life through metabolic programming and epigenetic mechanisms.

Summary Points

- Breastmilk carotenoids vary between different populations and geographical locations and according to circadian and within-feeding variations in breastmilk composition, maternal nutritional status, age, and premature birth.
- Breastmilk sample collection protocol for carotenoid analysis should take into account circadian and within-feeding variation in milk composition, data about infant feeding, and method of milk expression.
- During storage and handling breastmilk samples should be protected against oxygen, light, and temperature. Between collection and analysis samples should be stored in a very low temperature (−80 °C) for as short period of time as possible.
- The most popular method of breastmilk carotenoid analysis is HPLC or U-HPLC. Prior to analysis breastmilk samples should be saponificated, hydrolyzed, and extracted.
- The best protocol for collection of nutritional data is at least a 3-day food record and additional FFQ (if repeated records are not possible). Development of national databases of food carotenoids is necessary.

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Part III

Diets and Macronutrients

The Dietary Diversity Score

14

Methods, Indicators, and Applications to General Population

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Abstract

One of the main risk factors for several non-communicable diseases (NCDs) is improper dietary intake. The main item of healthy diet is food diversity. Diverse diets have all the food groups including grains, meat, vegetables, fruits, and dairy product. These diets provide nutrient adequacy and optimal growth and development. Dietary diversity is the consumption of various foods and food groups during a certain period. The Dietary Diversity Score (DDS) is an easy and practical index that is used in several studies to assess dietary quality of population in different age groups and countries. DDS is used to assess the diversity within food groups based on a healthy and balanced diet. Several studies showed that DDS could be used for the assessment of dietary diversity as a useful and practical indicator. It has been shown that a higher dietary diversity is correlated with improving diet quality.

Keywords

Food variety · Dietary diversity · Dietary indices · Diet quality indices

Abbreviations

CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DDS	Dietary diversity score
FAO	The Food and Agriculture Organization
HDDS	Household Dietary Diversity Score
IDDS	Individual Dietary Diversity Score
NCDs	Non-communicable diseases
WDDS	Women's Dietary Diversity Score

Introduction

In recent years, diet quality indicators have been developed to provide useful information about the association between diet and non-communicable diseases (NCDs). Urbanization and quick socioeconomic alteration lead to nutrition transition and increase some unhealthy dietary patterns including Western dietary pattern, particularly among children and adolescents. The low intake of fruit and vegetables, fiber-rich foods, and dairy products and the high intake of fatty, sugary, and convenience foods are unhealthy dietary patterns that are increasing (Gupta et al. 2020).

Nowadays, the quality of diets has increasingly interested rather than single food-based or single nutrient-based approaches. Different indices have been developed to reflect the quality of diets and the diversity of food group choices. These indices examine diet-disease relationships better than single nutrients or foods (Alkerwi et al. 2015). Some indices are based on specific dietary patterns, such as the Dietary Approaches to Stop Hypertension (DASH) diet and the Mediterranean diet. However, some indices have been developed according to various dietary components (Phillips et al. 2019).

Despite several indices for the assessment of diet quality, the best indicator for prediction of health outcomes has not yet identified (Burggraf et al. 2018). Several systematic reviews assessed variation in diet quality indices and their limitations (Dalwood et al. 2020; Trijsburg et al. 2019; Hlaing-Hlaing et al. 2020). However, more studies are needed to investigate the effectiveness of these indices in predicting health status, the risk of chronic diseases, or mortality and diet-disease relationships. Several studies have validated individual dietary indicators; however, few studies have assessed the relationship between diet quality indices and biomarkers of diet and disease (Dalwood et al. 2020; Asghari et al. 2017; Yang et al. 2019).

Dietary diversity reflects the variety of diet and distinct and wholesome foods. Dietary diversity promotes nutrient adequacy, high dietary quality, and the maintenance of optimal health. The *Dietary Guidelines for Americans* have recommended a variety of food groups particularly vegetables and protein-rich foods to improve public health and reduce the risk of several diet-related chronic disorders including metabolic syndrome (Yosaee et al. 2017), obesity (Golpour-Hamedani et al. 2020) and cardiovascular diseases (Farhangi and Jahangiry 2018; Ritter et al. 2021).

The Diet Diversity Score as Diet Quality Indicator

The Dietary Diversity Score (DDS) is a potential indicator of nutritional adequacy. It quantifies the number of food groups in a diet during a specific period (Golpour-Hamedani et al. 2020). Some evidence has shown that DDS is correlated and interacted with nutritional status (Khamis et al. 2019; Sié et al. 2018). Some confounding factors including urban or rural area and socioeconomic, demographic, and food group variation might lead to this contradiction (Agrawal et al. 2019; Morseth et al. 2017). In addition, different diagnostic interpretations of the results of correlation and variability of nutrient content in each food group may lead to various conclusions and inconsistencies (Habte and Krawinkel 2016).

There is no any definitive definition of DDS among studies. The definition differs with the aim of the study and the outcome. DDS is defined as eating varieties of different and healthy foods that improve nutrient adequacy and dietary quality and maintain optimal health (Rawal et al. 2020; Golpour-Hamedani et al. 2020).

Dietary variety is recommended by the *Dietary Guidelines for Americans 2015–2020* that considers the diversity of nutrient-dense foods and the type of food intake particularly vegetables and protein-rich foods. It helps to increase biodiversity and sustainability and reduces the risk of various NCDs (Rawal et al. 2020).

Diet Diversity Score in Relation to Diet Quality

Dietary habits are associated with several NCDs including cardiovascular disease (CVD), cancer, and obesity (Kojima et al. 2020). Poor dietary habits are one of the most important lifestyles associated with NCDs. Diets rich in vegetables, legumes, whole grains, fish, and oils reduce the risk of NCDs higher than diets rich in red meat, added sugar, and salt. Sociodemographic factors and education level are

correlated with dietary habits and nutrient intake. Lower socioeconomic status is correlated with less nutrient-dense diets and lower intakes of vegetables, fruits, fish, and vitamins and minerals (Kojima et al. 2020; Sakurai et al. 2018).

Parental education is positively associated with healthier food habits among children (Ober et al. 2021). A national survey in Sweden showed adolescents with higher levels of parental education ate more vegetables and fish and less sugar-sweetened beverages. In addition, they consumed more nutrients including vitamin D, iodine, and iron. Dietary patterns measure the multidimensional aspects of food intake. Understanding the effect of the overall diet on health outcomes can be difficult by assessment of single nutrient or food items. Several diet indices have been developed to investigate the quality of diet. Another dimension of diet is variation. DDS is based on the food group number or food item number consumed. Better overall composition of nutrient is associated with a high DDS (Moraeus et al. 2020).

Poor diet diversity is one of the most important reasons of micronutrient deficiency. Therefore, a key element of quality of diets is dietary diversity and consumption of a variety of foods according to several nutritional guidelines. The consumption of a wide variety of healthy foods is recommended to ensure that there are high-quality dietary patterns especially in developing countries. Healthy dietary patterns contain balanced and varied diet including fresh and natural foods, fruits and vegetables, and foods with vitamins and minerals (Meng et al. 2018; Rahmannia et al. 2019).

Dietary diversity can be categorized based on foods and food groups, nutrients only, or foods and nutrients. Dietary diversity according to food groups and some nutrients is more correlated with micronutrient deficiencies in comparison with individual nutrients or foods. However, mostly the Dietary Diversity Score is calculated by the number of food groups consumed during a specified time (Rahmannia et al. 2019; Brazier et al. 2020; Geng et al. 2018).

DDS can be calculated at the household or individual level. A measure of access to food is considered for the calculation of DDS at the household level. Dietary diversity reflects quality and diet micronutrient adequacy at the individual level. DDS can predict nutrient adequacy of vulnerable age groups, introduce or promote certain food groups, and can be considered as an advocacy tool for more attention to the vulnerable communities (Zhao et al. 2017).

Dietary Diversity and Nutrient Adequacy

Micronutrient malnutrition, overweight, and obesity are the most serious nutritional concerns in all age ranges and different countries. They have long-term effects on incidence and development of NCDs. There are complex lifestyles, dietary patterns, and nutrition transitions in many countries. Thus, more attention should be paid to both undernutrition and overnutrition and their complications (Zhao et al. 2017; Mak et al. 2019).

Most of the required micronutrients are provided by daily diet. So, consumption of a balanced diet containing diverse food is important. The variety of foods may ensure adequate intake of nutrients and other useful substances. Several studies showed positive correlation between dietary diversity and micronutrients adequacy particularly in pediatric age groups in developing countries (Mallard et al. 2016; Zhao et al. 2017).

The absence of dietary diversity may lead to many problems in developing countries in that diets are predominantly based on starchy foods, low animal products, and few fresh fruits and vegetables. These plant-based diets usually have a low bioavailability and lead to deficiency in micronutrients including zinc (Islam et al. 2015).

Studies have demonstrated that an increase in an individual DDS is correlated with an increase in diet nutrient adequacy in various age groups. DDS has been validated for many age and sex groups. DDS has been directly associated with sufficient micronutrient density of complementary foods for infants and young children (Modjadji et al. 2020; Kennedy et al. 2007a), adolescents (Isabirye et al. 2020), and adults (Gómez et al. 2020; Zhang et al. 2017).

Findings of the Third National Health and Nutrition Examination Survey on 8719 subjects reported that DDS was positively associated with serum levels of nutrients including vitamins C, E, and folate and all carotenoids, with the exception of lycopene. These nutrients oppositely correlated with body mass index, serum homocysteine, C-reactive protein, plasma glucose, and hemoglobin A1C as biomarkers of disease risks (Kant and Graubard 2005).

Seasonality and the Dietary Diversity Score

There is seasonality in food availability in developing countries. This means that food is available in abundance at certain times of the year; however, there is very little food at other times. So, the food dietary diversity might differ with season. There are seasonal differences in the intake of fruits, legumes, roots, plantains, and some flour. For example, intake of vitamin A-rich foods increases in rainy season in which green leafy vegetables are abundant (Waswa et al. 2021).

Few studies have assessed the effect of seasonality on DDS, nutrient adequacy, and its determinants. There are not any consistent results about seasonal fluctuation, dietary diversity, and nutrient adequacy. One study showed a decrease in dietary diversity and nutrient adequacy in the lean season in comparison with the abundant season (Becquey et al. 2010). Dietary diversity increased because of intake of foods rich in proteins and vegetables during the pre-harvest period. In the rural Tanzanian community, intake of various foods in the lean and post-harvest seasons impacted on the consumption of some vitamins (Ayenew et al. 2018).

A study in Kenya showed that the intakes of calcium, zinc, iron, and folate during the rainy season were more than the dry season in preschool children (M'Kaibi et al. 2015). A study in Burkina Faso showed a greater micronutrient intake in the post-harvest season in comparison with the lean season. The results demonstrated a higher

DDS and a lower nutrient adequacy in the lean season in comparison with the post-harvest season in rural area (Becquey et al. 2012; Arsenault et al. 2014). These inconsistent findings show a low consistency in the association between seasonal fluctuations, dietary diversity, and nutrient adequacy. Thus, several studies are required to clarify the impact of seasonality on DDS (Abizari et al. 2017).

Calculation of the Dietary Diversity Score

Dietary quality includes variety and diversity. For calculation of dietary variety, the number of foods consumed is measured from a predetermined list during a specific time. The overall variety of foods consumed, adherence to dietary pattern recommendation for food groups, and their relative distribution are considered for dietary diversity. There are various ways of dividing food into several food groups. The latest Chinese Dietary Guidelines recommend the intake of at least 12 and 25 food groups per day and per week, respectively (Zhao et al. 2017).

There are different serving cutoff points to find the sufficient amount of intake of a specific food according to the variety or diversity score. These differences lead to inconsistent findings. Mirmiran et al. reported that intake of at least half a serving of a food can be considered as the diversity score (Mirmiran et al. 2004). Kennedy et al. reported that the variety score could be considered when at least 10 g of food was consumed (except fats and oils) (Kennedy et al. 2007b).

The Food and Agriculture Organization (FAO) recommended 2 DDS including the Household Dietary Diversity Score (HDDS) based on 12 food groups and the Women's Dietary Diversity Score (WDDS) as Individual Dietary Diversity Score (IDDS) based on 9 food groups. Twelve food groups that are considered in HDDS are cereals; white tubers and roots; vegetables; fruits; meat and eggs; fish and other seafood; legumes, nuts, and seeds; milk and milk products; oils and fats; sweets; spices and condiments; and beverages. Nine food groups that are considered in IDDS are starchy staples; dark green leafy vegetables; other vitamin A-rich fruits and vegetables; other fruits and vegetables; organ meat; meat and fish; eggs; legumes, nuts, and seeds; and milk and milk products (Islam et al. 2015).

Eight food groups were considered in the Children Dietary Diversity Score including grains, roots, and tubers; vitamin A-rich plant food; other fruits or vegetables; meat, poultry, fish, and seafood; eggs; pulses, legumes, and nuts; milk and milk products; and foods cooked in oil/fat (Islam et al. 2015).

According to studies, various methods have been used to calculate DDS. The usual and most widely used methods consider various foods or food groups consumed during a specific period. However, food and food group classification and the length of the specific period differ between the studies. The lack of discernment between healthful and unhealthful foods, individual food quantities distribution, and the lack of ability to find difference in particular nutrient contents are the limitations of count-based measures. Recently, for solving the limitations of the count-based approach, evenness (balance in the distribution of calories among individual foods)

and dissimilarity (health-related attributes of various food intakes) have also been considered (Rawal et al. 2020; Otto et al. 2015).

The Dietary Diversity Score and NCDs

The diversity in diets and a healthy balanced diet provide adequate nutrients during childhood, illnesses, and pregnancy. DDS has been considered as an indicator of diet quality that is correlated negatively with risk of NCDs and nutrition-related diseases including CVD, hypertension, diabetes mellitus, metabolic syndrome, cancers, osteoporosis, obesity, and abdominal adiposity. More micronutrients including fiber, vitamin C, and calcium are consumed in a diverse diet. These nutrients have beneficial effects on the prevention of NCDs (Gholizadeh et al. 2018).

Diet diversity in pregnancy decreases low birth weight risk (Rammohan et al. 2018). Breast milk cannot provide the enough energy and micronutrients for healthy development. So complementary feeding with sufficient diversity (both quality and quantity) is required during lactation. Diverse diets are associated with anthropometric status and hemoglobin concentrations (Shively and Evans 2021; Dewey and Adu-Afarwuah 2008).

Metabolic syndrome has cardio-metabolic risk factor components that its prevalence is increasing worldwide. Healthy dietary patterns, intake of various nutrients and food groups, and restriction of calorie can prevent the complications of metabolic syndrome. Several studies have assessed the impacts of single foods or nutrients on the components of metabolic syndrome (Abete et al. 2010; Syauqy et al. 2018). However, investigation of the effects of overall diet on metabolic syndrome may be more informative. Findings showed negative association between metabolic syndrome components and DDS in adults (Farhangi and Jahangiry 2018).

A balanced nutrient intake during lifetime can reduce morbidity and mortality (Otto et al. 2015). Intake of a higher number of major food groups decreases the risk of causes of mortality (Conklin et al. 2016). However, the findings of a multi-ethnic cohort study showed that consumption of a higher number of various food items (between 0 and 120) at least twice a week was not correlated with incidence of type 2 diabetes (Otto et al. 2015). According to the findings of the studies, it seems that diet diversity can be associated with nutrient intake, weight disorders, and some chronic disorders and can be correlated with quality of life (Fig. 1).

The Dietary Diversity Score and Anthropometric Measurements

Adequate and diverse diets have a positive influence on growth spurt, normal pubertal development, and education capacity of adolescents (Ochola and Masibo 2014).

A cross-sectional study among 384 high-school female students indicated that a higher DDS in adolescents might be correlated with a higher general health (Aminianfar et al. 2021). According to recommendation, adolescents should consume

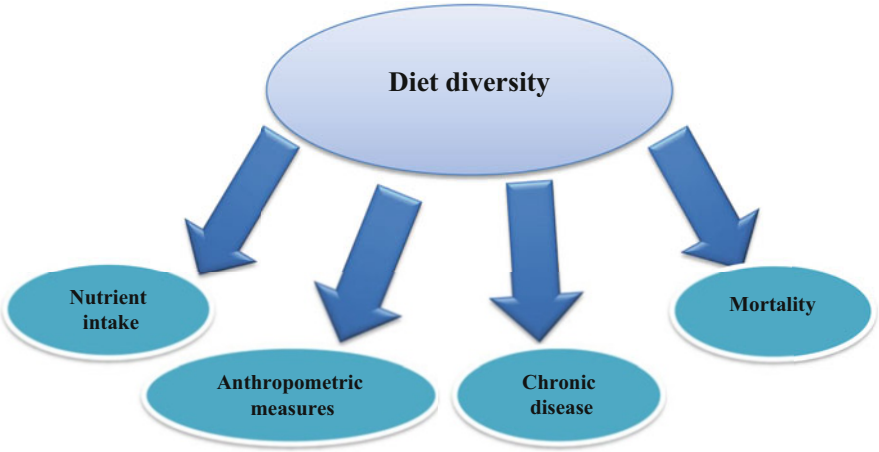

Fig. 1 Association between diet diversity and some parameters related to quality of life

various foods including low-fat animal products, fruits, vegetables, and whole grains; drink plenty of water; and consume a low amount of sugar, fat, and salt (Isabirye et al. 2020).

DDS is correlated with anthropometric measurements. There are inconsistent findings about dietary diversity and anthropometric status (Fernandez et al. 2016; Tiew et al. 2014). It has been shown that a high dietary diversity with unhealthy food items may be adversely associated with weight gain and obesity. However, a high dietary diversity through consumption of low-energy and nutrient-dense foods provides a low-calorie diet and prevents the increase of adipose tissue (Vadiveloo et al. 2015).

One study on children showed a positive (Fernandez et al. 2016) and another showed a negative (Hurley et al. 2009) association between dietary variety and diversity with annual change in BMI z-score. In childhood, DDS is associated with height-for-age, weight-for-height, weight-for-age, and mid-upper arm circumference (Shively and Evans 2021).

A higher DDS has been associated with reduced stunting. A study on children under 5 years old showed that children in the higher-diet-diversity group were 30% less likely to be stunted in comparison with children from the lower-diet-diversity group (Rah et al. 2010). A higher DDS has also been correlated with increased micronutrient consumption (Mak et al. 2019; Arsenault et al. 2013).

Our systematic review and meta-analysis did not show any significant relationship between DDS and body mass index, waist circumference, abdominal obesity, and overweight (Table 1) (Qorbani et al. 2021).

The World Health Organization considers DDS as one of the key indicators to investigate the practices of child feeding, nutritional status, and dietary adequacy (Amugsi et al. 2017). However, socioeconomic status, health service availability, water sanitation, and parental education are important factors in child nutritional

Table 1 Meta-analysis of association between diet diversity score and anthropometric measures

	Effect size (95% CI)	Model
Obesity	0.97 (0.58, 1.35)[a]	Random
Overweight	1.03 (0.73, 1.31)[a]	Random
Abdominal obesity	1.17 (0.6, 1.73)[a]	Random
Body mass index	0.32(−0.01, 0.65)[b]	Random
Waist circumference	0.05(−0.31, 0.41)[b]	Random

[a]OR (95% CI)
[b]SMD (95% CI)

status. So, more studies are required to explain the potential factors that are associated with child nutrition in various cultural backgrounds (Yang et al. 2019).

Some foods with high levels of fats, added sugars, and sodium are recommended to be consumed moderately. A higher variety of these foods is correlated with a higher prevalence of obesity (Vadiveloo et al. 2013).

The findings of a recent systematic review showed that DDS had the highest frequency in developing countries (Asghari et al. 2017). Another study on healthy females showed that a higher DDS reduced the risk of general obesity and abdominal obesity approximately 80% (Azadbakht and Esmaillzadeh 2011). There are some reasons for these inconsistent findings. First, a higher food variety is correlated with a higher energy intake, and so may be related with general obesity. Second, a higher food variety can be enhanced by intake of various healthy and low-energy-dense food groups including vegetables, whole grains, and fruits that reduce general obesity risk (Asghari et al. 2017). A lower dietary quality may lead to insufficient essential nutrient intake and finally malnutrition, particularly in childhood. Enough nutrient consumption for optimal growth and development is needed (McDonald et al. 2015).

Applications to Prognosis, Other Diseases, or Conditions

According to findings moderate increases in diet quality that is assessed by some indices such as DDS are correlated with a decreased risk of NCDs including CVD, diabetes, and musculoskeletal, respiratory, and mental disorders (Lagström et al. 2020).

However, the effects of diet quality on diseases depend on the etiology of diseases. Inverse association was reported between a high diet quality and coronary heart disease, stroke, and heart failure. The findings of meta-analyses showed that healthy or prudent dietary patterns that contain healthy foods could prevent stroke and heart failure (d'Almeida et al. 2018; Iacoviello et al. 2018). DDS can be correlated with psychological disorders. A cross-sectional study on 360 women aged 20–49 showed that DDS could be inversely correlated with depression in women (Poorrezaeian et al. 2017).

Recent findings reported an opposite relationship between high diet quality and healthy dietary patterns and risk of colorectal cancer. A meta-analysis of cohort studies confirmed the beneficial effects of diet quality on gastrointestinal cancers

(Feng et al. 2017). According to the findings of a systematic review, a high diet quality decreased hepatocellular and prostate cancers risks (Potter et al. 2016). DDS is correlated with blood antioxidant markers. Enhancing the dietary diversity might be correlated with reduction of oxidative stress (Narmaki et al. 2015). In addition quality of diet could be associated with neurodegenerative and cognitive diseases. A study on women aged 20–50 years showed that a higher DDS was correlated with better visual and auditory sustained attention (Shiraseb et al. 2016). The highest diet quality is inversely correlated with all-cause mortality risk (Morze et al. 2020). A meta-analysis of observational studies showed that there was no significant relationship between DDS and cardio-metabolic risk factors. However, there was a significant association between DDS and TG statistically (Qorbani et al. 2021). A study on children and adolescents showed that a higher DDS was correlated with higher anthropometric indices and risk of obesity (Heidari-Beni et al. 2021). Contradictory results confirm the importance of new and valid DDS standard tools as a criterion of diet quality.

Conclusion

Dietary habits are modifiable risk factors, and improving them should be a priority of public health. According to the previous findings, it is suggested that DDS can be considered as a beneficial, simple, and convenient tool for assessment of dietary diversity, micronutrient adequacy of the diet, and further nutrition-related diseases. Higher education levels were associated with higher healthy diet diversity. So, at the individual and community levels, nutrition education related to dietary diversity and consumption of a variety of healthy foods should be emphasized.

Summary Points

- DDS is a potential indicator of nutritional adequacy.
- DDS decreases the risk of several NCDs.
- Nutrient adequacy can be predicted by DDS.
- Diverse and healthy diets provide sufficient nutrients during childhood, illnesses, and pregnancy.
- Dietary diversity, diet micronutrient adequacy, and nutrition-related diseases can be determined by DDS as a beneficial, simple, and convenient tool.

Key Facts of DDS

- The quality intake of several foods and food groups during a certain period can be determined by dietary diversity.
- DDS is considered as an easy and practical index for assessment of dietary quality of diet in various age groups and countries.

- DDS assesses the variety within food groups based on a healthy and balanced diet.
- Adequate and diverse diets have beneficial effects on the prevention of NCDs.

Mini-dictionary of Terms

- **Dietary diversity**: It is defined as the number of different foods or food groups consumed over a given reference period.
- **Dietary Diversity Score**: It is a potential indicator of nutritional adequacy. It quantifies the number of food groups in a diet during a specific period.
- **Household Dietary Diversity Score**: It is used for the measurement of household food access. Household food access is defined as the ability to obtain an adequate quality and quantity of food to meet all household members' nutritional requirements for productive lives.
- **Individual Dietary Diversity Score**: It is a simplified method for assessing the quality of diets, defined as the number of food groups represented in the diet over a period of time.
- **Non-communicable diseases**: They include heart disease, stroke, cancer, diabetes, and chronic lung disease. The increasing incidence of NCDs is due to four major risk factors: tobacco use, physical inactivity, the harmful use of alcohol, and unhealthy diets.

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Dietary Phytochemical Index as a Biomarker in Nutritional Studies: Features and Applications

15

The Dietary Phytochemical Index

Mostafa Qorbani, Pooneh Angoorani, and Hanieh-Sadat Ejtahed

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Abstract

Phytochemicals are bioactive nutrient plant chemicals that may provide desirable health benefits beyond basic nutrition to reduce the risk of major non-communicable diseases. Phytochemical content in different dietary patterns can be evaluated by the dietary phytochemical index (DPI). DPI is defined as the percent of dietary calories derived from foods rich in phytochemicals including fruits, vegetables, legumes, nuts, seeds, whole grains, and foods compounded therefrom. There is some evidence assessing the effects of dietary quality via DPI on the risk of different non-communicable diseases. In this chapter, we evaluated the health protection effect of phytochemical-rich diets using DPI as a dietary biomarker in nutritional epidemiology and summarized the results of studies that investigated the association between DPI and various disorders including obesity, metabolic syndrome, cardiovascular disease, cancers, and neurological disorders.

Keywords

Phytochemical · Dietary phytochemical index · Non-communicable diseases · Obesity · Metabolic syndrome · Cardiovascular disease · Cancer · Neurological disorders

Abbreviations

AhR	Aryl hydrocarbon receptor
BIA	Body adiposity index
BMI	Body mass index
CIs	Confidence intervals
CVD	Cardiovascular disease
DII	Dietary inflammatory index
DPI	Dietary phytochemical index
EDIP	Empirically developed dietary inflammatory potential
FBG	Fasting blood glucose
FFQ	Food frequency questionnaire
HbA1C	Hemoglobin A1C
HDL-C	High-density lipoprotein cholesterol
IL-6	Interleukin-6
LDL-C	Low-density lipoprotein cholesterol
Med-DQI	Mediterranean dietary quality index
miRNA	Micro-ribonucleic acid
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCDs	Non-communicable diseases
nrf2	Nuclear factor erythroid 2-related factor 2
OGTT	Oral glucose tolerance test
ORs	Odds ratios
RNA	Ribonucleic acid

ROS	Reactive oxygen species
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumor necrosis factor-α
WC	Waist circumference
WHO	World Health Organization

Introduction

The increasing prevalence of non-communicable diseases (NCDs) – a variety of disorders such as cardiovascular diseases, diabetes, and cancer – is becoming a global health concern and imposes economic burden on all countries (Heller et al. 2019). The role of dietary factors and nutritional regimens in the prevention of NCDs and its progression has been extensively studied, and numerous reports suggested the positive role of phytochemical-rich diets in this context (Estruch et al. 2013; Wang et al. 2016). Phytochemicals are defined as bioactive nutrient plant chemicals in fruits, vegetables, grains, and other plant foods that may provide desirable health benefits beyond basic nutrition to reduce the risk of major chronic diseases (Liu et al. 2004). These components have great antioxidant potential and modulation role in detoxifying enzyme expression or activity which can reduce the risk of various diseases linked with oxidative damage (Cieślik et al. 2006). The dietary phytochemical index (DPI) is an easy and practical method to determine the phytochemical content of the foods in clinical practice (McCarty 2004). There are some documents assessing the relationship between the dietary quality via DPI and different diseases. In this chapter, we intend to evaluate the health protection effect of phytochemical-rich diets using DPI as a dietary biomarker.

Sources and Types of Phytochemicals

Wide-ranging dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs, and spices. Broccoli, cabbage, carrots, onions, garlic, whole wheat bread, tomatoes, grapes, cherries, strawberries, raspberries, beans, and soy foods are also the common sources of phytochemicals. Phytochemicals accumulate in different parts of the plants, such as in the root, stem, leaf, flower, fruit, and seed (Koche et al. 2016). Many phytochemicals, particularly the pigment molecules like anthocyanins and flavonoids, are often concentrated in the outer layers of various plant parts like the leaves and fruits of vegetables. However, the levels of these phytochemicals vary from plant to plant depending upon the variety and climatic growing conditions (Rao 2003). The exact classification of phytochemicals has not been given so far, because of their diverse forms and structures. Classically, the phytochemicals have been classified as primary or secondary metabolites, depending on their role in plant metabolism. Primary metabolites include the common sugars, amino acids, proteins, purines, and

pyrimidines of nucleic acids, chlorophylls, etc. Secondary metabolites are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumins, saponins, phenolics, and glucosides (Fig. 1). Literature survey indicates that phenolics are the most common and structurally diverse plant chemicals (Ramawat et al. 2009; Hahn 1998). The percent occurrence of phytochemicals in the plants and their role in human healthcare are given in Table 1.

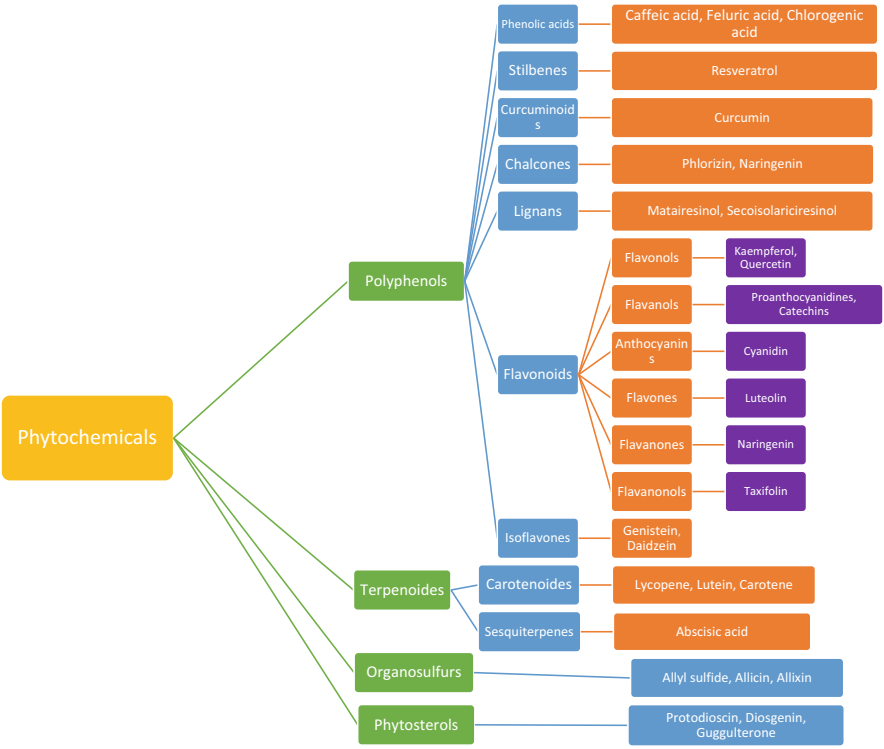

Fig. 1 Classification of dietary phytochemicals

Table 1 Occurrence and role of major classes of phytochemicals

Class of phytochemicals	Occurrence as natural product (%)	Role in healthcare
Phenolics	45	Antioxidants, anticancerous, cytotoxicants, antimicrobials, and vasodilating
Terpenoids and steroids	27	Antimicrobials, detoxifying agents, strengtheners, antirheumatics, antimalarial, hepaticidal
Alkaloids	18	Neuropharmaceuticals, anticancerous, sedatives, antimicrobials, insecticidal
Other chemicals	10	Anti-inflammatory, immunostimulating

Definition and Utility of "Phytochemical Index"

Dietary phytochemical content can be evaluated by dietary phytochemical index (DPI), first proposed by McCarty (McCarty 2004). DPI is defined as the percent of dietary calories derived from foods rich in phytochemicals including fruits, vegetables (not including potatoes, but including other tubers), legumes, nuts, seeds, whole grains, and other foods compounded there. Fruit and vegetable juices, although lacking fiber and some of the phytochemical content of rinds or peels, are often rich in phytochemicals and thus should be counted in the index. Soy protein, though evidently not a whole food, is usually a good source of isoflavones and thus should be counted. Extra-virgin olive oil is relatively rich in absorbable antioxidants and thus might be given "partial credit," but most other oils used in cooking, although containing some fat-soluble phytochemicals, are low in phytochemicals on a per-calorie basis and thus should be excluded from this index (McCarty 2004; Vissers et al. 2002). Meanwhile, in some studies, tea (Mottaghi et al. 2015), wine, beer and cider (Vincent et al. 2010), and seaweed (Kim and Park 2020; Im et al. 2020) were also classified as phytochemical-rich foods. This index is a simple method for the assessment of phytochemical intake which, despite its limitations, could provide important background for diet quality and may have high practical and clinical uses (Vincent et al. 2010). Epidemiologists could use DPI as a very rough index of total dietary phytochemical content and attempt to correlate DPI with health outcomes. It is evident that the phytochemicals derived from a variety of foods can interact in additive and perhaps sometimes synergistic ways to modulate physiological function; this principle would be acknowledged by the use of DPI in epidemiological investigations (Franceschi et al. 2001; Augustin et al. 2001; Salmeron et al. 1997; Salmerón et al. 1997; Liu et al. 2000, 2002). DPI would also be of use to clinical nutritionists, as a tool for analyzing the quality of their clients' diets, for encouraging their clients to eat more phytochemical-rich foods, and for quantifying the extent of their clients' progress in this regard. DPI can be viewed as a useful tool for aiding assessment of the health impacts of diets high in phytochemical-rich plant foods and for encouraging the consumption of such diets. Theoretically, a vegan diet that excluded refined grains, potato products, hard liquors, and added sugars and oils could have a DPI of 100. However, the DPI content of most current diets is much lower than this which means that should be improved by increasing intakes of fibers, vitamins, trace minerals, and plant proteins while decreasing sources of animal fats and proteins (McCarty 2004).

DPI is calculated based on [daily energy derived from phytochemical-rich foods kJ (kcal)/total daily energy intake kJ (kcal)] × 100 (McCarty 2004). Practically, to use DPI in studies, dietary data should be collected through food frequency questionnaire (FFQ) by asking participants to designate their consumption frequency for each food item over a specified period of time on a daily, weekly, or monthly basis. Totally, FFQ checklist is a dietary assessment tool which consists of a list of foods and beverages and collects portion size information in its semiquantitative format. The reliability and validity of FFQs should be evaluated in different populations.

There is no fixed and definite value or cutoff that indicates the desired amount of DPI, so in studies, the range of DPI is expressed according to tertile or quartile

categories. For example, in Tehran Lipid and Glucose Study, the range of DPI in the first, second, third, and fourth quartiles of population was <20.6, 20.6–28.1, 28.2–36.9, and >36.9%, respectively (Mirmiran et al. 2012b).

The Mediterranean diet is the best example of a phytochemical-rich diet (Fig. 2). The Mediterranean dietary pattern is characterized by a high intake of vegetables, legumes, fruits and nuts, cereals, and olive oil but a low intake of saturated lipids, a moderately high intake of fish, a low-to-moderate intake of dairy products (mostly in the form of cheese or yoghurt), a low intake of meat and poultry, and a regular but moderate intake of alcohol (Tur et al. 2005). The Mediterranean diet is considered as one of the healthiest dietary patterns, and numerous epidemiological and nutritional studies have shown that Mediterranean countries benefit from higher life expectancy and lower rates of mortality caused by chronic diseases (Mariscal-Arcas et al. 2009). This dietary pattern exerts most of its health-promoting effects via its bioactive compounds including phytochemicals. These phenolic ingredients mostly concentrated in olives, grapes, and nuts protect against cardiovascular events, oxidative

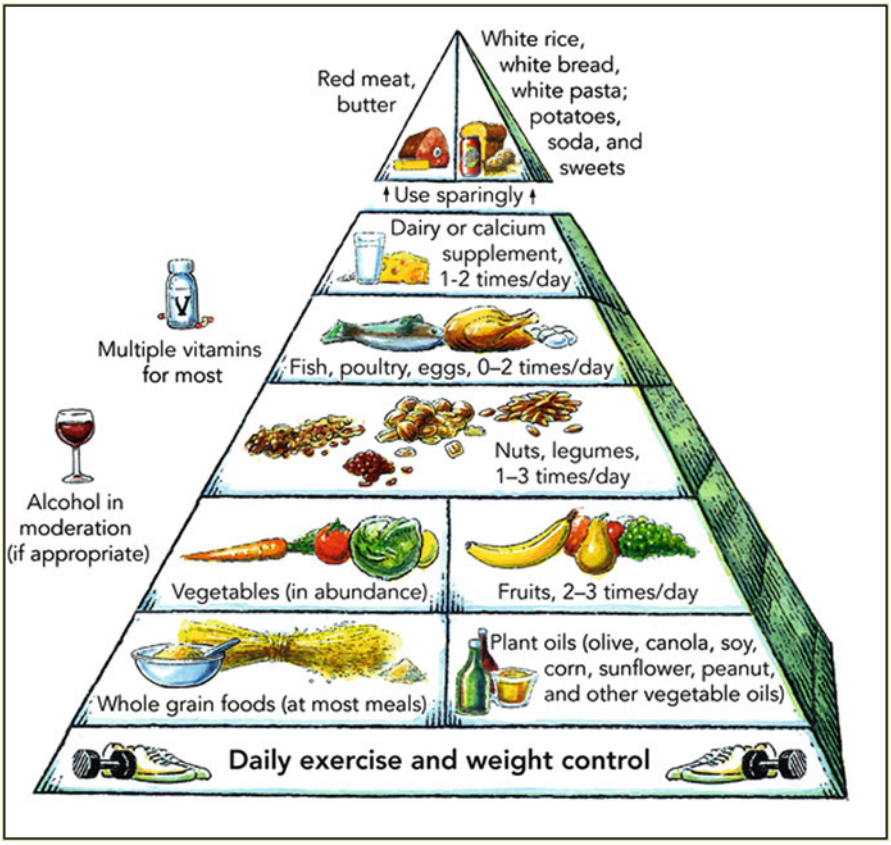

Fig. 2 The Mediterranean diet pyramid

stress, and vascular dysfunction (Carluccio et al. 2003; Mottaghi et al. 2015). The recommendations made by the National Research Council and the American Heart Association regarding diet and health are based on consumption of 30% or less of the daily total energy from fat, 10% or less of the total energy from saturated fat, 30 mg/d or less from cholesterol, 55% of energy from complex carbohydrates, and 5 servings or more from fruits and vegetables (Gerber et al. 2000), and it seems that the Mediterranean dietary pattern could cover these recommendations sufficiently.

Application of Dietary Phytochemical Index as a Biomarker for Non-communicable Diseases (Fig. 3)

Phytochemical Index, Obesity, and Metabolic Disorders

Since obesity and related metabolic disorders have become one of the major public health challenges worldwide, various nutritional epidemiological studies have been conducted to identify key dietary factors affecting the risk of obesity and metabolic

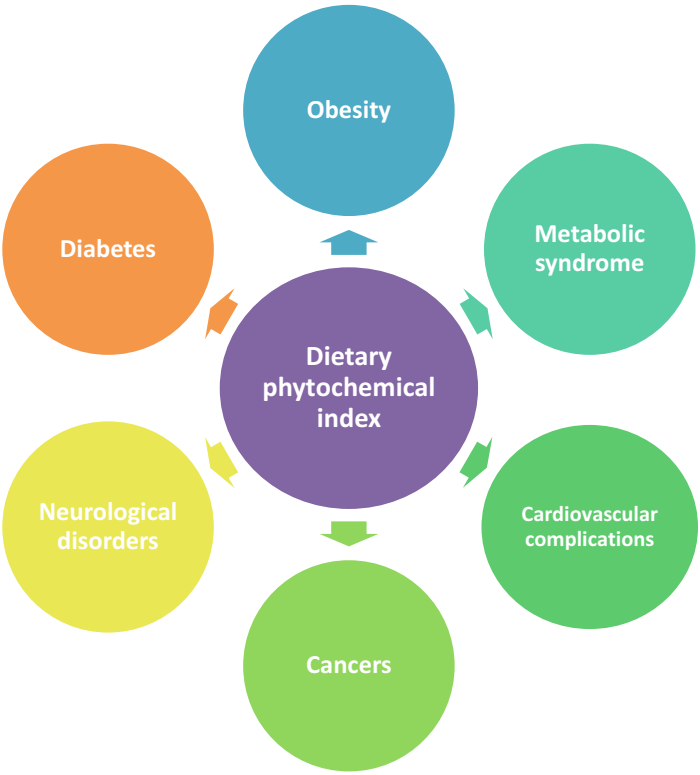

Fig. 3 Application of dietary phytochemical index in nutritional epidemiology

syndrome. Consuming a diet rich in vegetables, fruits, and whole grains has been shown to be effective for weight control (He et al. 2004; Liu et al. 2003; Serdula et al. 1996). DPI has previously been proposed to evaluate the effect of phytochemicals on the prevention of obesity and metabolic disorders in epidemiological studies. A cross-sectional study of 356 children aged 7–10 years in Iran showed a negative association between phytochemical intake and obesity (Eslami et al. 2020). In a longitudinal study, DPI was negatively associated with 3-year changes in waist circumference (WC) among Iranian population (Mottaghi et al. 2015). Secondary analysis of these data showed a significant adverse relationship between the highest quartile of DPI and 3-year changes in body weight and body adiposity index (BAI), but not in waist circumference (Mirmiran et al. 2012a). In another Iranian study on 235 patients with type 2 diabetes, a significant reverse correlation was observed between body mass index (BMI) of patients and DPI (Ziaee et al. 2021). The Tehran Lipid and Glucose Study involving 2567 adults reported that the participants in the highest DPI quartile had a 66% lower risk of abdominal obesity than those in the lowest quartile (Bahadoran et al. 2013a). A cross-sectional study on 54 American overweight young adults (18–30 years old) showed significant adverse links between DPI and body weight, body mass index (BMI), WC, waist-to-hip ratio, and body fat percentage (Vincent et al. 2010). In a Korea National Health and Nutrition Examination Survey, the association between the DPI and obesity/abdominal obesity was examined on 57,940 adults. DPI was calculated using the 24 h recall data, and multivariable-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using logistic regression models. After multivariable adjustment, a higher DPI was found to be associated with a lower prevalence of obesity and abdominal obesity (Im et al. 2020).

In addition to obesity, the correlations between DPI and metabolic syndrome and its components, including hypertension, insulin resistance, and disorders of lipid profiles, have been investigated. In a public survey on 31,319 Korean adults (aged $\geq$19 years), after adjusting for multiple confounding variables, participants in the highest DPI quintile had significantly lower prevalence of abdominal obesity, hyperglycemia, high blood pressure, hypertriglyceridemia, and metabolic syndrome (Kim and Park 2020). In a cross-sectional study in Iran, the association between the DPI and metabolic syndrome was examined among 850 adults (aged 18–65 years). The findings demonstrated an inverse association between DPI and increased risk of central obesity, but no significant association was observed between DPI and other components of the metabolic syndrome (Firouzabadi et al. 2021). In a longitudinal study among Iranian population, an inverse association was found between DPI and changes in triglycerides (TGs) and total cholesterol (TC) in men but not in women (Golzarand et al. 2014). Furthermore, DPI was found to be inversely related to 3-year changes in blood triglycerides and lipid accumulation product in 1552 Iranian population in another longitudinal investigation (Mottaghi et al. 2015). In a study conducted on 261 diabetic patients (aged 18–35 years), after adjustment for potential confounders, participants in the highest tertile of DPI showed 98% lower chance of low-density lipoprotein/high-density lipoprotein cholesterol (LDL-C/HDL-C) ratio compared with those in the lowest tertile (Aghdam et al. 2021). Moreover, in an

Iranian study carried out on 235 patients with type 2 diabetes (aged 30–65 years), a significant positive correlation was observed between DPI and HDL but not with LDL, TC, and TG (Ziaee et al. 2021). However, the results of the study conducted on American overweight young adults did not show any correlation between DPI and serum cholesterol subtractions (Vincent et al. 2010).

The association between DPI and blood sugar and insulin resistance has been also investigated. It seems that a higher DPI can decrease the risk of prediabetes (Abshirini et al. 2018) and also has potential protective effects against the development of insulin resistance (Bahadoran et al. 2015). In this regard, a case-control study conducted among Iranian individuals revealed that participants in the higher quartiles of DPI had lower fasting blood glucose (FBG) and oral glucose tolerance test (OGTT) and the DPI score was inversely related to prediabetes. This means that participants in the upper quartile had a lower OR for prediabetes in comparison with those in the lowest quartile (Abshirini et al. 2018). Accordingly, following a high DPI program was associated with a lower risk of hyperinsulinemia, insulin resistance, and insulin insensitivity in a longitudinal research among Iranian communities, but not with a marker of B cell dysfunction (Bahadoran et al. 2015). In contrast, DPI was not significantly correlated with the levels of hemoglobin A1c (HbA1C) and blood glucose in a small sample size study of overweight young adults in the USA (Vincent et al. 2010) which is in line with the results of another study (Ziaee et al. 2021).

The associations between DPI and blood pressure were also investigated in a few studies. Although the studies demonstrated the positive effect of diets with greater amount of vegetables and phytochemical-rich foods on blood pressure (Saleh et al. 2020), no significant correlation was found between DPI and systolic and diastolic blood pressure in studies (Aghdam et al. 2021; Ziaee et al. 2021).

There is no clear mechanism by which DPI may affect body weight and metabolic components. However, some evidences derived from in vitro and in vivo studies revealed that phytochemicals may decrease adiposity and body fat through various ways, some of which include targeting adipocyte lifecycle such as a higher induction of apoptosis and inhibiting proliferation (Abshirini et al. 2018), elevating lipolysis and insulin sensitivity followed by a reduction in lipogenesis and angiogenesis, and decreasing pro-inflammatory mediators in adipocytes (Lee et al. 2011). Moreover, based on a longitudinal evidence, it is suggested that consuming nearly 40% of dietary energy intake from phytochemical-rich foods could be protective against weight gain (Mirmiran et al. 2012a). Accordingly, it seems that adherence to a higher DPI is related to a lower dietary fat intake and a higher dietary fiber and protein as well as reduced total dietary energy intake (Bahadoran et al. 2013a, 2015; Mirmiran et al. 2012a; Golzarand et al. 2014; Mottaghi et al. 2015). Furthermore, another explanation for reducing risk of obesity through high intake of phytochemical-rich foods could be its lower glycemic index (Abshirini et al. 2018). So apparently a higher DPI might be effective on individual's weight management.

Regarding lipid profile, it has been proposed that phytosterols as a subclass of phytochemicals are responsible for phytochemical-related blood lipid improvement (Katan et al. 2003; Demonty et al. 2009). Competing with cholesterol for micellar

incorporation, phytosterols can lower cholesterol level. Besides to inhibition of intestinal cholesterol uptake (Hallikainen et al. 2000), some phytochemicals can be considered a potent ligand for lipid metabolism regulatory receptors such as peroxisome proliferator-activated receptors (PPAR). By upregulating genes involved in fatty acid transport and peroxisomal and mitochondrial oxidation, these receptors can modulate lipid metabolism (Ko et al. 2010). Moreover, some researchers attributed the serum lipid reduction to flavonoids, such as quercetin (Mirmiran et al. 2009). The synthesis of triglyceride (TG) can be inhibited by quercetin at the human intestinal CaCo-2 cell line, through inhibiting the secretion of apoB-100 and apoB-48 (Casaschi et al. 2002). In addition, galangin, as another type of flavonoids, can reduce serum lipid levels, lipid peroxidation, and hepatic lipid accumulation such as TGs (Kumar and Alagawadi 2013). Due to the lack of evidence about other lipid profile such as total cholesterol, it is not possible to make a general conclusion.

As mentioned earlier, there is contradictory evidence regarding the association of DPI with glucose homeostasis. Nevertheless, some evidences showed that phytochemicals, especially polyphenols, can improve insulin resistance by regulating insulin production and secretion as well as pancreatic β-cell protection. Likewise, these phytochemicals derived from plant foods have been highlighted as a potential therapeutic factor for enhancing uptake of insulin-dependent glucose transporter 4 (GLUT4). It is thought that eating more fruits with high polyphenol content, such as whole fruits like blueberries, grapes, and apples, is linked to a lower risk of type 2 diabetes (Bahadoran et al. 2013c).

With regard to high blood pressure, as a risk factor for cardio-metabolic disorders in the current study, no evidence was existed regarding association between hypertension and PI. Although, there is not any consensus on the relationship between DPI and high blood pressure, several probable mechanisms have been suggested for the antihypertensive properties of phytochemicals. An increasing body of evidence revealed that the etiology of hypertension is complicated by oxidative stress, which results in an excessive production of reactive oxygen species (ROS) (Moline et al. 2000). Phytochemicals are the key components of the diet that have antioxidant properties, which reduce oxidative stress and help prevent hypertension (Boeing et al. 2012). The augmentation of endothelial nitric oxide synthase activity and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which results in vascular smooth muscle tone relaxation, is another underlying mechanism by which phytochemicals can ameliorate high blood pressure (Steffen et al. 2008).

In summary, phytochemical-rich diets and a high DPI score have positive effects on general and abdominal obesity, dyslipidemia, insulin resistance, and metabolic syndrome. However, large-scale prospective cohort studies and randomized clinical trials are needed to confirm these findings.

Phytochemical Index and Cardiovascular Diseases

Cardiovascular disease (CVD) is one of the most common causes of morbidity and mortality in different communities accounting for more than 31% deaths worldwide;

more than 75% of these deaths occur in low- and middle-income countries. According to the reports of the World Health Organization (WHO), an estimated 17.7 million people died from CVD in 2015, representing 31% of all global deaths. Of these deaths, an estimated 7.4 million were due to coronary heart disease, and 6.7 million were due to stroke (Organization 2017). The treatment costs of CVD imposed on individual and society are substantial. Since cardiovascular diseases affect the majority of young working and productive population especially in low-income countries, its heavy financial burden in these countries is alarming (Clark et al. 2009). These issues highlight the need for more attention to deal with the impact of CVD in the following decades. The primary prevention of CVD is an important priority for health policy development; healthy dietary habits, smoking cessation, weight management, regular physical activity, and stress management all are most important strategies to reduce the risk of CVD later in life (Stewart et al. 2017). The role of dietary factors and nutritional regimens in the prevention and progression of CVD has been extensively studied; numerous reports suggested the role of healthy dietary choices and higher intakes of healthy foods rich in dietary antioxidants including fruits and vegetables in the prevention and treatment of cardiovascular events (Farhangi et al. 2017). Recently, new dietary indices have been developed and attracted much attention because of capturing multiple dietary factors and providing a comprehensive assessment of diet quality, accounting for the complex interactions between nutrients and foods. Because of the pro-inflammatory and anti-inflammatory roles of nutrients, the inflammatory potential of diet could be associated with numerous inflammation-associated diseases including CVDs. Besides DPI, other indices including the Mediterranean dietary quality index (Med-DQI), dietary inflammatory index (DII), and empirically developed dietary inflammatory potential (EDIP) have been developed for assessing dietary phytochemical content and inflammatory or anti-inflammatory potential of diet (Tabung et al. 2016). It has been shown that DII and EDIP were negatively associated with vitamin E and total dietary antioxidant scores and also were in negative association with DPI and Med-DQI in patients (Abbasalizad Farhangi and Najafi 2018; Tabung et al. 2017). Several previous studies suggested the protective role of phytochemical-rich diets such as the Mediterranean dietary regimen in the prevention of cardiovascular events (Estruch et al. 2013; Fraser et al. 2008). The Tehran Lipid and Glucose Study involving 2567 subjects aged 19–70 years reported that participants in the upper quartiles of DPI had lower risk of CVD risk factors (Bahadoran et al. 2013a). Moreover, patients with lower phytochemical intakes had significantly higher Med-DQI scores (Farhangi et al. 2017). Evidences have shown that the Mediterranean and phytochemical-rich diets both reduce total cholesterol, LDL-C, and non-HDL-C levels and have significant cardioprotective effects (Lukaczer et al. 2006). The role of the Mediterranean dietary pattern in the primary prevention of CVD in a sample of 7747 adults at high risk of CVD but without a manifest disease has been established in a large interventional study before; in a multicenter trial in Spain, a Mediterranean diet supplemented with extra-virgin olive oil and a Mediterranean diet supplemented with mixed nuts reduced the risk of major cardiovascular diseases by 30% and 28%, respectively (Estruch et al. 2013). In a meta-analysis, intervention

with a Mediterranean diet was associated with a 38% relative reduction in the risk of CVD clinical events (Martinez-Gonzalez and Bes-Rastrollo 2014). It has been suggested that there would be a synergy among the nutrient-rich foods included in the Mediterranean diet that fosters favorable changes in intermediate pathways of cardio-metabolic risk factors, like hyperlipidemia, insulin sensitivity, resistance to oxidation, inflammation, and vaso-reactivity (Jacobs Jr et al. 2009). Furthermore, it was shown that a two-point increase of the Mediterranean dietary score was associated with 33% reduced risk of mortality from cardiovascular causes (Trichopoulou et al. 2003). Due to the high DPI of the Mediterranean diet, these findings confirm the positive role of phytochemicals in CVDs. In fact, over the past decades, numerous dietary models have been proposed to protect against metabolic abnormalities, and among them, only the phytochemical-rich diets with a high DPI such as the Mediterranean diet demonstrated a beneficial effect (Sofi and Casini 2014).

Since oxidative stress is strongly correlated with CVD pathogenesis, numerous trials have attempted to assess the clinical value of antioxidant-rich foods in cardiac patients. Phytochemical-rich diets have a great antioxidant potential and modulation role in detoxifying enzyme expression or activity which can reduce the risk of various diseases linked with oxidative damage (Cieślik et al. 2006). Animal studies have demonstrated that intake of phytochemical-rich diet can reduced hypertension-associated diastolic dysfunction (Seymour et al. 2008, 2013). The cardiac-specific mechanisms associated with these phytochemical-related benefits may be due to reduced cardiac oxidative damage and fibrosis that contribute to adverse changes in cardiac geometry and hemodynamic function. It was suggested that a higher intake of phytochemical-containing foods may achieve cardiac benefits that isolated antioxidant supplements may not (De Lorgeril et al. 2001; Witte and Clark 2005). Studies in animal and cell culture models show that phytochemical-rich diets activate the expression of Phase I/II metabolism in varied body tissues (Moskaug et al. 2005; Murray 2006; Moon et al. 2006). This effect of phytochemical rich diet could be achieved by ligand interaction or by altering kinase signaling cascades that then activation of transcription factor such as aryl hydrocarbon receptor (AhR) and NF-E2 related factor (nrf2) increase the transcription/translation of genes related to Phase I/II metabolism which affect cellular responses to oxidative stress (Seymour et al. 2013). Another possible mechanism associated with the phytochemical-related benefits is increased glutathione reserve following phytochemical intake. Among several proteins involved in antioxidant defense, glutathione is abundant in the heart and plays an important role in cardioprotection and heart failure (Myhrstad et al. 2002; Moskaug et al. 2005; Masella et al. 2005).

In summary, phytochemical-rich diets could alter the cardiac transcriptome in ways that favor greater resistance to prolonged hypertension and concomitant oxidative stress.

Phytochemical Index and Cancer

Cancer is a major public health problem that has a significant global impact on both developed and developing countries. In 2018, an estimated 18.1 million new cases of

cancer occurred worldwide which are likely to increase to 23.6 million new cases each year by 2030 (Bray et al. 2018). Phytochemicals are becoming increasingly important sources of anticancer compounds which can prevent cancer initiation, promotion, and progression by direct antioxidant activity, anti-inflammatory potential, and modulation of key cellular signaling pathways (Tan et al. 2011). Phytochemicals used in cancer treatment may reduce adverse side effects and help treat cancer, and they have shown promising anticancer efficiency in recent years against different types of cancer like colon, pancreatic, prostate, and breast cancers by various mechanisms (Carter et al. 2004; Donald et al. 2012; Marconett et al. 2010; Ouhtit et al. 2013). Although the association of each whole-plant phytochemical-rich foods such as whole grains, legumes, fruits, vegetables, soy products, and nuts with the risk of cancer has been investigated, there is limited evidence regarding this association in the new frame as DPI. The DPI in relation to the risk of breast cancer was assessed only in a few studies. The results from a case-control study on 100 breast cancer cases showed that increased energy intakes from phytochemical-rich foods (more than 30% of energy per each 1000 kcal), independent of confounding variables, were related to the decreased risk of breast cancer. The risk of breast cancer in the fourth quartile of DPI (higher intake of phytochemical-rich foods) was significantly decreased compared to the first quartile (OR = 0.08; 95% CI = 0.01–0.84) (Bahadoran et al. 2013b). In another case-control study of 115 subjects with benign breast diseases, it was shown that after adjustment for confounding variables, a higher DPI score was related to lower benign breast diseases (OR = 0.3; 95% CI = 0.12–0.93) (Aghababayan et al. 2020). Another study evaluated the DPI in relation to the risk of glioma (Rigi et al. 2021). Glioma refers to all tumors that are supposed to originate from neuroglial cells. Glioma is the most prevalent brain tumor, and almost 77% of all brain malignant tumors are attributed to glioma (Organization 2018). This study was conducted on 128 newly diagnosed cases of glioma and 256 age- and sex-matched controls. The findings showed that individuals in the top tertile of DPI were more likely to be older and female. Before taking potential confounders into account, subjects in the top tertile of DPI tended to have a 40% reduced chance of glioma than those in the bottom tertile (OR = 0.60; 95% CI: 0.35–1.02). After controlling for age, sex, energy intake, several demographic variables, and dietary intakes, the association between DPI and glioma became strengthened (OR = 0.43; 95% CI: 0.19–0.97) (Rigi et al. 2021). Phytochemicals can prevent cancer initiation, promotion, and progression by direct antioxidant activity, anti-inflammatory potential, and modulation of key cellular signaling pathways (Shu et al. 2010). They possess complementary and overlapping mechanisms to slow down the carcinogenic process by scavenging free radicals, suppressing survival and proliferation of malignant cells, as well as diminishing invasiveness and angiogenesis of tumors (Lu et al. 2018). They exert a wide and complex range of actions on different molecular targets and signal transduction pathways including membrane receptors, kinases, downstream tumor-activator or tumor-suppressor proteins, and transcriptional factors (Choudhari et al. 2020). Phytochemicals could act as the potent preventive compounds in the development of mammary carcinoma through several mechanisms including the ability to inhibit aberrant proliferation in initiated

and transformed cells, regulate cell-cycle progression, induce cellular apoptosis, increase the formation of anti-proliferative estradiol metabolite, and inhibit cell migration and metastasis (Sun and Liu 2008). Another possible mechanism is the role of phytochemicals in regulating microRNAs (miRNAs) which regulate the expression of genes involved in development, growth, proliferation, and apoptosis (Ventura and Jacks 2009). Recent reports demonstrate that phytochemical compounds play an important role in the prevention and treatment of breast cancer by regulating specific miRNAs (Tilghman et al. 2013).

In conclusion, these findings reveal an inverse association between DPI and the risk of breast cancer, independent of potential confounding variables. Therefore, DPI as a simple method for assessment of phytochemical intake could provide important background regarding diet quality in relation to all kinds of cancers.

Phytochemical Index and Neurological Disorders

Neurological disorders could result in/from structural, biochemical, or electrical abnormalities in the brain, spinal cord, or other nerves (Zis and Hadjivassiliou 2019). Mental problems such as depression and anxiety are among the most common neurological disorders in both developed and developing countries (Steel et al. 2014). Major depression and anxiety affect about 4.7 and 7.3% of people worldwide, respectively (Ferrari et al. 2013).

Evidences reveal that foods rich in phytochemical compounds have been associated with a lower risk of neurological and mental health disorders (Hosseinzadeh et al. 2016). However, there are limited publications regarding DPI and neurological disorders. A cross-sectional study conducted on 488 women (aged 20–50 years) showed that the women in the highest tertile of DPI had a lower prevalence of depressive symptoms (OR = 0.22; 95% CI: 0.12, 0.38) and anxiety (OR = 0.33; 95% CI: 0.20, 0.55), as well as psychological distress (OR = 0.30; 95% CI: 0.18, 0.49), compared with those in the lowest tertile (Mofrad et al. 2019).

There are some probable mechanisms that explain the association between DPI and neurological disorders. Studies have shown that dietary patterns characterized by high intakes of fruits, vegetables, whole grains, olive oil, fish, low-fat dairy products, and antioxidants and a low content of animal foods have been associated with a lower odds of neurological disorders (Li et al. 2017; Payne et al. 2012). It is demonstrated that the DPI is positively correlated with decreased oxidative stress (Vincent et al. 2010). In many cases, increased oxidative stress was observed in neurological disorders (Martínez et al. 2010; Spaas and Van Veggel 2021). Oxidative stress, which is the imbalance between the production of reactive oxygen species and antioxidant defenses, is one of the main pathogenesis mechanism causes of brain cell death in neurological disorders (Niedzielska et al. 2016). Phytochemical components have a great antioxidant potential and modulation role in detoxifying enzyme expression or activity which can reduce the risk of various diseases linked with oxidative damage including brain and neurological disorders (Cieślik et al. 2006). Recent studies have shown that some

phytochemicals could inhibit monoamine oxidase activity and mitochondrial enzymes that catalyze the oxidation of monoamines including serotonin, norepinephrine, and dopamine (Grosso et al. 2013). The use of these natural antioxidants plays an important role in preventing neurodegeneration through maintaining the redox homeostasis (Trinh et al. 2021). Another possible mechanism is the anti-inflammatory effect of phytochemicals. Some studies demonstrated that inflammation involved in pathogenesis of mental health disorders (Howren et al. 2009). Phytochemicals have anti-inflammatory activity by decreasing biomarkers of inflammation, including TNF-α and IL-6, inhibiting neuronal growth especially in the hippocampus, and modification of gastrointestinal bacteria composition. Phytochemicals could increase mRNA expression of several peptides that seems to be dysregulated in neurodegenerative diseases (Dash et al. 2015; Rendeiro et al. 2015). Moreover, phytochemicals increase extracellular signal-regulated kinase phosphorylation, a main component of the neurotrophic and specific signaling pathways participating in neuronal differentiation and plasticity (Xiong et al. 2011; Bahramsoltani et al. 2015).

In summary, consumption of foods rich in phytochemicals is associated with a lower risk of neurological disorders, and DPI as a novel index showing the amount of phytochemical-rich food intake can be a good candidate for this evaluation. These initial findings regarding the relationship between DPI and mental health need to be confirmed with prospective studies.

Limitations of DPI and Concluding Remarks

DPI can be viewed as a practical and useful tool for specified evaluation of the health impacts of diets high in phytochemicals, but as a quantitative measure of phytochemical intake, DPI has some evident weaknesses. First of all, it does not acknowledge the contribution made by the consumption of noncaloric foods such as green or black tea. Besides, it does not take into consideration the fact that the phytochemical-to-calorie ratio of plant foods varies a great deal. Furthermore, it is doubtless true that the health-promoting utility of certain phytochemicals is greater than that of others; however, the DPI does not take this into account. Thus, two diets with identical DPI rating could result in various health outcomes owing to marked differences in the quantity or quality of their phytochemicals. Moreover, the bioavailability of phytochemicals is dependent to gut microbiota composition, and it could affect the effectiveness of phytochemicals consumed. However, this issue is not considered in this index and can overshadow the performance of this index as a biomarker.

Totally, a few studies have been conducted regarding the health impact of this index in different racial populations, so there is no specific value or cutoff point that indicates the desired amount of DPI for using in clinical settings. Therefore, further investigation is required in order to use this index as a biomarker in nutritional epidemiology contributing toward the evidence used in guiding dietary recommendations for prevention of various non-communicable diseases.

Mini-dictionary of Terms

- **Cardiovascular diseases**: A class of diseases that involve the heart or blood vessels.
- **Dietary phytochemical index (DPI)**: The percent of dietary calories derived from foods rich in phytochemicals.
- **Glioma**: All tumors that are supposed to originate from neuroglial cells (non-neuronal cells in the central nervous system).
- **Isoflavones**: Plant-derived compounds with estrogenic activity.
- **Mediterranean diet**: A traditional dietary pattern in Mediterranean countries, characterized especially by a high consumption of vegetables and olive oil and moderate consumption of protein and thought to confer health benefits.
- **Metabolic syndrome**: A cluster of biochemical and physiological abnormalities associated with the development of cardiovascular disease and type 2 diabetes.
- **Neurological disorders**: Structural, biochemical, or electrical abnormalities in the brain, spinal cord, or other nerves which can result in a range of symptoms.
- **Phytochemicals**: Bioactive nutrient plant chemicals in fruits, vegetables, grains, and other plant foods that may provide desirable health benefits beyond basic nutrition to reduce the risk of major chronic diseases.

Key Points of Dietary Phytochemical Index (DPI)

- DPI is defined as the percent of dietary calories derived from foods rich in phytochemicals.
- Phytochemical-rich foods include fruits, vegetables (not including potatoes, but including other tubers), legumes, nuts, seeds, and whole grains.
- DPI is calculated based on daily energy derived from phytochemical-rich foods kJ (kcal)/total daily energy intake kJ (kcal)] $\times$ 100.
- Theoretically, a vegan diet that excluded refined grains, potato products, hard liquors, and added sugars and oils could have a DPI of 100.
- Further investigation is required in order to use this index as a biomarker in nutritional epidemiology.

Summary Points

- Phytochemicals have a great antioxidant potential and modulation roles in detoxifying enzyme expression or activity.
- Phytochemicals can reduce the risk of various diseases linked to oxidative damage.
- Dietary phytochemical index (DPI) can be viewed as a simple tool for specified evaluation of the health impacts of diets high in phytochemicals.

- DPI content of most current diets is very low and should be improved by increasing intakes of fibers, vitamins, trace minerals, and plant proteins while decreasing consumption of animal fat and animal protein.
- A higher DPI is negatively associated with obesity, metabolic syndrome, cardiovascular diseases, breast cancer, and neurological disorders.
- Few studies have been conducted regarding the health impact of this index in different racial populations, so there is no specific value or cutoff point that indicates the desired amount of DPI for use in clinical settings.

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Mediterranean Diet Adherence and Serum Markers of Lipids

16

Ioannis-Nektarios Elmaliklis and Antonios Koutelidakis

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Abstract

The majority of recent research data has underlined that diet influences the serum lipid profile, especially triglycerides (TGs), total cholesterol (TC), low-density lipoproteins (LDL), high-density lipoproteins (HDL), and very-low-density lipoproteins (VLDL), lipid peroxidation (LPO), and a number of other parameters such as apolipoproteins (Apo), lipoprotein a (LP(a)), C-reactive protein, systolic blood pressure (SBP), diastolic blood pressure (DBP), etc., having a direct impact on human health. In the above context, there is a scientific viewpoint about the association of Mediterranean diet with serum markers of lipids and human health. The Mediterranean dietary pattern is characterized by an abundant use of olive oil; a high consumption of plant-based foods (fruits, vegetables, legumes, grains, nuts, seeds, etc.); frequent but moderate wine consumption (mainly red) at meals; moderate intake of fish, seafood, fermented dairy products (yogurt, cheese, milk), poultry, and eggs; and reduced consumption of red meat, processed meat products, and also sweets.

Several studies, including the most recent meta-analyses, show that the traditional Mediterranean diet, defined as the dietary pattern of the individuals that live around the Mediterranean Sea in the 1960s, may be the most effective dietary pattern with protective effect on chronic diseases (cardiovascular, obesity, metabolic syndrome, etc.) attributed to the association of the above type of diet with serum markers of lipids. Taking this for granted, researchers indicated health benefits related to possible positive changes on serum markers of lipids, in people with a high adherence to the Mediterranean dietary pattern, while higher risk of chronic diseases correlated with the possible adverse effects of serum lipid markers in those with a low adherence to the above type of diet. In addition, it is necessary to explain more the relationship of Mediterranean diet adherence with serum markers of lipids and consequently the health outcomes with a view to laying a solid foundation for further research on this topic in the future.

Keywords

Mediterranean diet adherence · Health effect · Serum markers of lipids · Health outcomes · Cardiovascular diseases

Abbreviations

Apo (A1)	Apolipoprotein A1
Apo (B)	Apolipoprotein B
C12:0	Lauric acid
C14:0	Myristic acid
C16:0	Palmitic acid
CRP	C-reactive protein
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid

HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
LDL	Low-density lipoprotein
LF	Low fat
LP(a)	Lipoprotein (a)
LPO	Lipid peroxidation
SBP	Systolic blood pressure
SDLDL	Small dense low-density lipoprotein
TAC	Total antioxidant capacity
TC	Total cholesterol
TGs	Triglycerides
VLDL	Very-low-density lipoprotein

Introduction

Serum Lipid Profile

The major lipids present in the plasma are fatty acids, TGs, cholesterol, and phospholipids and other lipid-soluble substances, present in much smaller amounts (Burdge and Calder 2015). More specifically, serum lipid profile is a pattern of lipids in the blood, typically including the levels of TC, HDL-C, the calculated LDL-C, VLDL-C, and TGs. Along with the above lipid profile, it should be noted and a number of other parameters (total lipids, Apo, LP(a), etc.) for its most complete description and analysis (Orozco-Beltran et al. 2017).

Association of Serum Lipid Profile with Human Health

Numerous studies worldwide have clearly indicated that the abnormal levels of serum lipids are associated with a high risk of appearance of various chronic diseases. Dyslipidemia is a disorder characterized by abnormal amounts of blood lipids and lipoproteins and one of the top five major risk factors leading to cardiovascular disorders (Ding et al. 2016). A great example is that a part of incidents with cardiovascular diseases globally is related not only to high blood cholesterol levels but also to the form of lipoproteins, as oxidized and very-low-density lipoproteins are more atherogenic, independently whether cholesterol levels are increased (Vincent-Baudry et al. 2005).

Particularly, elevated LDL-C and TGs and decreased concentrations of HDL-C are associated with an increased risk of coronary heart disease and atherosclerosis (Yuan et al. 2007). Apart from cardiovascular diseases, diabetes (owing to abnormal blood sugar levels) and obesity (due to high blood cholesterol levels) are associated with aggravated lipid profile, concluding that an adverse lipid profile is related to the induction of a large part of disease states (Franz et al. 2003; Dallongeville et al. 2007).

Influence of Lipid Profile by Environmental Factors, Such as Nutritional Attitudes

Undoubtedly, a normal serum lipid profile implies the improved host health. The formation of serum markers of lipids in general population that is extremely associated with health is influenced not only by genetic background (Pires et al. 2016) but also by a plethora of environmental parameters related to the general lifestyle. In the last decades, various secondary factors such as lifestyle, social, economic, and mainly dietary factors have been investigated as possible parameters with a significant effect on specific biomarkers, especially glucose levels, serum markers of lipids, postprandial state, etc. They have also been correlated with obesity, cancer, diabetes, and cardiovascular disease (Puolakka et al. 2016). The above factors are of high significance in individuals with elevated serum lipids (Harada-Shiba et al. 2018).

Focusing on dietary factors, a vertical increase of various chronic diseases has occurred in recent years due to the high adherence to Western type of diet that is associated with a high risk of dyslipidemia, accompanied by a decrease of HDL-C and a raise in TC, LDL-C, and VLDL-C levels (Sadakane et al. 2008; Sialvera et al. 2012). Thus, care should be taken to avoid unhealthy eating habits, limiting the appearance of negative health outcomes associated with dyslipidemia which is an index of abnormal serum markers of lipids. A healthy diet adherence combined with a balanced lifestyle is a significant strategic treatment for non-normal blood lipids that are negatively associated with human health (Yoon 2014).

Particularly, research data clarifies the crucial role of a balanced diet based on the Mediterranean dietary pattern with a wide variety of dietary nutrients derived from the consumption of specific functional foods, including also a decreased intake of saturated fat, low cholesterol and simple sugar consumption, and a high intake of complex carbohydrates without reducing protein intake, in order to regulate the serum markers of lipids and thus prevent dyslipidemia or other pathological conditions. This protective effect may be associated with a decreased risk of atherosclerosis or other cardiovascular disease development (Kwiterovich Jr 2008).

Association of Dietary Nutrients (Fats, Carbohydrates, Proteins, Fibers, and Antioxidants) with Serum Lipid Profile

A large part of studies has been conducted in order to investigate the association of different dietary nutrients with lipid profile (Reiner et al. 2011). The mechanisms of nutrient effect on serum lipids are indicated below:

Fats

Although previous recommendations about the dietary treatment of hypercholesterolemia focused on the decrease of total dietary fats to less than 30% of daily calories, this strategy did not bring the expected results in improving the lipid profile. Also the replacing of fats with carbohydrates tends to reduce the HDL levels, inducing

hypertriglyceridemia (Howard et al. 2006), and diets rich in fats raise the total intake of calories, increasing the risk of various diseases such as obesity and atherosclerosis (Fletcher et al. 2005; Mensink et al. 2003). Taking into account the above data, it is observed that the most important factor which in turn affects the lipid profile is the composition of fat intake with emphasis on mono-unsaturated fats as they are associated with positive changes on serum markers of lipids (Hu et al. 2001).

Saturated Fatty Acids

Saturated fats contained in red meat, butter, or dairy products (milk, yogurt, and cheese) are dietary nutrients with an active effect on the LDL-C levels, because they increase the amount of cholesterol in all lipoprotein fractions when they replace other dietary fatty acids or carbohydrates. A significant element is that the increase or decrease by 1% of caloric intake of saturated fats leads to increase or decrease respectively by 2% of LDL. The main fats in order for hypercholesterolemic activity are myristic (C14:0), palmitic (C16:0, and lauric (C12:0). It is also noted that a reduction of dietary fat intake has a beneficial effect on serum lipid profile, especially when accompanied by weight loss where required (Hu et al. 2001; Muller et al. 2003).

Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids contained in fish and fish oils have a protective effect against coronary heart disease and sudden death. Their benefits have a wide range and relate to lipid metabolism, lipoproteins, arterial contractility, endothelial function, and mechanisms of atherogenic process. Furthermore, omega-3 fatty acids have antiseptic and anti-inflammatory action, with docosahexaenoic acid and eicosapentaenoic acid to reduce the blood triglyceride concentrations but no low-density lipoprotein cholesterol levels. In addition to omega-3s, omega-6s are also included in polyunsaturated fatty acids and contained mainly in vegetable oils. Replacing saturated fats by omega-6 fatty acids (especially by linoleic acid: C18:2) leads to decreased LDL levels and also reduced HDL concentrations which should normally be high at increased levels. In general, n-6 polyunsaturated and n-3 polyunsaturated fatty acids have beneficial effect on serum markers of lipids which in turn may be associated with a low risk of numerous chronic diseases (Sabate and Ratzin-Turner 2001).

Monounsaturated Fatty Acids

Research data has shown that when monounsaturated acids, mainly oleic acid (C18:1) contained in olive oil, replace the dietary saturated fatty acids, there is a significant reduction of TC and LDL-C levels and blood triglyceride concentrations (Mensink et al. 2003). Also, oleic acid is the only monounsaturated fatty acid that is considered nutritionally significant. Common sources of oleic acid are olive and canola oil, as well as nuts. It has been proved that monounsaturated fats may reduce susceptibility of LDL to oxidation (Koutelidakis and Dimou 2016).

Furthermore, the action of monounsaturated fatty acids (cis-isomers) in HDL-C is influenced by the total fat intake, and the levels of HDL-C are raised when the high

intake of monounsaturated fat (>15%) is accompanied by moderate total intake (≈30%). On the other hand, the high total fat intake (>35% of the total kcal) does not seem to influence or increase slightly HDL-C concentrations when it is combined with a high intake of monounsaturated fatty acids (>15%). In the case of trans-isomers (resulting from industrial hydrogenation of the unsaturated fatty acids with the aim of solidifying and containing them in milk, butter, and beef fat), their effects are completely different and coincide with the effects of saturated fats as they increase the levels of LDL and decrease the concentrations of HDL, taking them at a level of 3 and 6% of the total consumed, respectively (Rosa Cde et al. 2015).

Dietary Cholesterol

Dietary cholesterol increases the TC/HDL-C ratio and also the levels of LDL-C but to a lesser extent than saturated fats (especially an increase of 25 mg leads to an increase of 1 mg/dl). Almost whether the dietary cholesterol intake exceeds the limit of 500 mg per day, the response of serum levels decreases, referring to individuals with normal sensitivity to dietary cholesterol due to the compensatory mechanisms developed in endogenous production. Also extremely important is the synergistic effect of saturated fats and dietary cholesterol on the increase of LDL-C levels, inducing the appearance of chronic diseases, most notably atherosclerosis (De Caterina et al. 2006; Fletcher et al. 2005).

Carbohydrates

The role of carbohydrates in serum lipid profile varies depending on the type of nutrient it replaces. When carbohydrates are consumed in place of saturated fats, they generally reduce the LDL-C levels. However, when replacement is not gradual, hypertriglyceridemia can be observed owing to the increased composition of VLDL-C and reduced lipoprotein lipase activity (decreased chylomicron catabolism and VLDL-C). Moreover, the type of carbohydrates that will replace fat (simple, complex, or fiber) is of great importance for the outcome of the lipid profile, with particular preference to a high consumption of complex carbohydrates or fiber that is more efficient in improving it (Ma et al. 2006).

Proteins

Generally, the role of protein intake is relatively neutral in terms of lipoprotein levels. Some studies conclude that low-fat diets decrease the risk of cardiovascular diseases (coronary heart disease, etc.) as they improve dyslipidemia (abnormal blood lipid concentrations). Also, the above results are due to the low intake of animal fat and not to the reduction of protein itself (Rosa Cde et al. 2015).

Fibers

The water-soluble fibers seem to have an effect in fat absorption and in serum cholesterol levels owing to the absorption of fatty acids, cholesterol, and bile acids in the fibers, their inability to form micelles to be absorbed by the small intestine, and their excretion through the feces or their degradation by colon bacteria. Depending on these mechanisms, on the one hand, the resorption of bile acids and reduced

cholesterol synthesis are required, and on the other hand the production of propionic acid by bacteria inhibits the cholesterol synthesis. Thus, a decrease of TC and LDL-C levels and especially an increase of plant water-soluble fibers by 5–10 mg per day can reduce the low-density cholesterol by 5%. Some of the most efficient soluble fibers are psyllium, pectins, guar gum, oatmeal and then oat bran, or soybean fiber, while the bran of corn, wheat, and rice do not have a significant effect on serum lipid profile (Rosa Cde et al. 2015).

Also, soluble dietary fibers including pectin from apples and citrus fruits, beta-glucans from oat and barley, and the fibers of flax and barley are well investigated as factors that decrease serum LDL-C. Cholesterol reduction is attributed to the binding of bile acids and inhibition of cholesterol synthesis. As fiber pass through the gastrointestinal system, it may create complexes which bind bile acids, transferring them from the intestines to the rest of the gastrointestinal tract. This process reduces the formation of bile acid micelles that are responsible for the absorption of cholesterol, preventing cholesterol absorption (Koutelidakis and Dimou 2016).

Research data has not proven until now the effect of these insoluble fibers on lipid profile. It should be noted that food consumed provide a mixture of soluble and insoluble fibers, and so the adequate consumption of fruits, vegetables, and whole grains can contribute to the improvement of hypercholesterolemia. Simultaneously, a high-fiber and low-fat meal is less energy dense with lower fat or added sugars, reducing the risk of chronic disease appearance (Anderson and Woodend 2003).

Furthermore, whole grain products may have a beneficial effect on serum markers of lipids. In particular, a meta-analysis showed an inverse association between TC, LDL-C (reduced), and HDL-C (increased), after consumption of glucans (oat, barley, and other functional foods). An additional meta-analysis proved that intake of 2–10 g per day of dietary soluble fiber from oats resulted in a small but important decrease of TC and LDL (Koutelidakis and Dimou 2016).

Antioxidants

A variety of antioxidants such as vitamins, minerals, carotenoids, and polyphenols have the ability to neutralize free radicals and reduce the LDL-C oxidation, improving the serum lipid profile and thus possibly acting as protective factors against mainly cardiovascular diseases. Nevertheless, further research should be conducted to draw safer conclusions about the effect of these dietary nutrients on the serum lipid profile (Dragsted et al. 2006; De Madeco Goncalves Fronta et al. 2010; Jenkins et al. 2011).

The Mediterranean Diet and Serum Markers of Lipids

Definition and Main Characteristics of the Mediterranean Diet

The Mediterranean diet is a dietary model that is consistent with the above dietary recommendations for the treatment of various abnormal lipid concentrations in the blood plasma. The above traditional dietary type refers to specific food consumption

patterns typical of some Mediterranean regions in the early 1960s, such as Spain, southern Italy, Crete, and other parts of Greece (Elmaliklis et al. 2019). Generally, the traditional Mediterranean diet is characterized by a high consumption of olive oil, fruits, vegetables, cereals, potatoes, nuts, legumes, and lean fish; a moderate consumption of milk, dairy products, poultry, and red wine; and finally a low consumption of red meat (Lampropoulou et al. 2020). In this dietary model, the most basic foods are olive oil, which is the main component of this diet and is a rich source of unsaturated fats, and also cereals and nuts, which are typically Mediterranean foods with a high content of nutrients, antioxidants, phytochemicals, and polyunsaturated fats.

This dietary pattern consists of:

- Daily consumption of non-refined cereals and products (whole grain bread, pasta, brown rice, etc.), vegetables (2–3 servings/day), fruits (6 servings/day), olive oil (as the main added fat component), and dairy products (1–2 servings/day, of which yogurt and cheese intake is higher than milk consumption)
- Weekly consumption of fish (4–5 servings/week), poultry (3–4 servings/week), and olives, pulses, and nuts (3 servings/week)
- Monthly consumption of red meat and meat products (4–5 servings/month) (Dontas et al. 2007) (Fig. 1)

Interaction of the Mediterranean Diet Adherence with Various Biomarkers (Including Lipid Markers) and Consequently Health Outcomes

The above dietary pattern has been recognized as a possible protective factor against various diseases, mainly in the prevention of cardiovascular diseases (Trichopoulou et al. 2009). Until now research data has demonstrated a possible protective effect of various specific functional foods included to the Mediterranean diet against atherosclerotic process and thus cardiovascular disease development.

Particularly, specific functional foods of the Mediterranean diet such as olive oil, honey, fruits (citrus fruits, pomegranate, grapes, berries), vegetables (tomatoes, cauliflower, broccoli, crumbled vegetables), wild greens (fennel, radish), and herbs (oregano, mint, salvia, sage, verbena, mountain tea) may have a positive effect on cardiovascular disease prevention, owing to the bioactive compounds of the above types of foods, as phytochemicals and polyphenols, such as oleuropein, resveratrol, sulforaphane, anthocyanins, quercetin, tannins, etc. Possible mechanisms of their action encompass improving serum lipid profile, endothelial factors, thrombotic factors, etc. Recent scientific data indicated that the Mediterranean diet adherence may be a significant health-promoting dietary approach for people of all ages (Koutelidakis and Dimou 2016).

In human subjects, a great part of studies indicated that a higher compliance with the Mediterranean diet was related to a higher physical activity, a lower body mass index and psychological stress or psychological disorders (anxiety and depression),

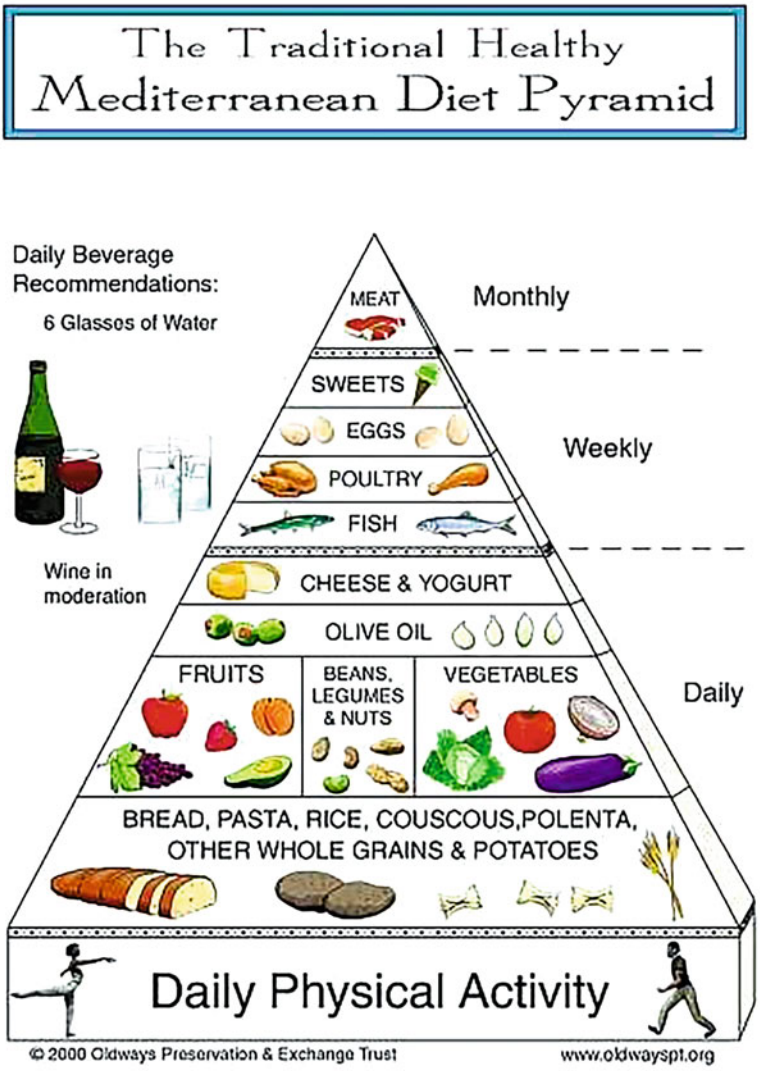

Fig. 1 The traditional healthy Mediterranean diet pyramid (Roycor 2017)

fewer subclinical atherosclerosis markers, improved metabolic biomarkers, and better blood pressure regulation and serum lipid profile. Understanding all the above information about individuals adhering to the Mediterranean diet, people's Mediterranean dietary habits in combination with a healthy lifestyle is of high importance to regulating serum markers of lipids, having thus positive health outcomes, and reducing pathological conditions associated with an abnormal lipid profile (Farajian and Zampelas 2015).

The main purpose of this chapter was to extend reliable scientific evidence about the association of Mediterranean diet adherence with the serum markers of lipids for its in-depth analysis and understanding.

Summary of Research Findings About the Effect of the Mediterranean Diet on Serum Lipid Profile and Health

Lyon Diet Heart Study

Researchers from the Lyon Diet Heart Study examining 605 patients aged 55–80 years with a history of myocardial infarction indicated the benefits of the Mediterranean diet extended to the secondary prevention of cardiovascular disease owing to improved serum lipid profile influenced by the above dietary type. In particular, volunteers with a high Mediterranean diet adherence had 50–70% lower risk of recurrent heart disease relative to those with a low adherence to this diet. According to the above findings, the potential significance of the Mediterranean dietary model compared with other recommended diets was attributed to the positive effect of the above diet on basic parameters of serum lipids, especially a decrease of TC, LDL-C, and TGs and conversely an increase of HDL-C, reducing thus the risk of cardiovascular or other severe chronic diseases (De Lorgeril et al. 1999).

HALE Study

The HALE study which was conducted on a sample of 2339 people (1507 men and 832 women) aged 70–90 years found that participants who did not follow a low-risk lifestyle, characterized by Mediterranean dietary habits, moderate alcohol consumption, increased physical activity, and abstinence from smoking, were at a greater risk of coronary heart disease and generally cardiovascular diseases. This higher risk was attributed to the adverse lipid profile of these individuals owing to their unbalanced eating habits, while the lower risk of the above diseases was associated with an improved serum lipid profile related to Mediterranean diet adherence and healthful lifestyle (Knoops et al. 2004).

ATTICA Study

In the same year, another research demonstrated a significant association of Mediterranean diet with serum lipid profile. Particularly in ATTICA study of 2282 individuals, 1128 men and 1154 women (aged >18 years old) from Greece, researchers observed that volunteers who adopted the Mediterranean diet and received statin had on average 9% lower TC, 19% lower LDL-C concentrations, and 32% lower oxidized LDL-C levels relative to those who were untreated and followed a Westernized diet, concluding on the one hand a beneficial lipid profile in volunteers with a high Mediterranean diet adherence and on the other hand an unhealthy serum lipid profile in those with a low Mediterranean dietary adherence who consumed foods found in Western dietary pattern (Panagiotakos et al. 2004).

The above results were in accordance with the findings of another research in 3042 healthy participants (1514 men and 1528 women) without clinical evidence of

cardiovascular disease selected from the Attica area of Greece. The objective of this study was to investigate the effect of the Mediterranean diet on the total antioxidant capacity. According to the results, volunteers who were more adherent to the Mediterranean diet had on average 11% higher total antioxidant capacity levels relative to participants with a lower adherence to the above dietary pattern. Also, the total antioxidant capacity was positively related to the consumption of olive oil and fruit and vegetables, whereas it was inversely correlated with the intake of red meat. An additional finding was that participants with the highest Mediterranean diet adherence had on average 19% lower oxidized LDL-C levels compared with those with the lowest. Therefore, greater adherence to the Mediterranean diet is associated with elevated total antioxidant capacity levels and a low oxidized LDL-C concentration, which may explain the beneficial role of this diet on the cardiovascular system (Pitsavos et al. 2005).

Medi-RIVAGE Study

A similar type of research, especially the Medi-RIVAGE study in 212 volunteers of moderate risk for cardiovascular diseases, comparing a 3-month dietary intervention with the Mediterranean diet and low-fat diet concluded that serum lipid profile, body mass index, and glucose levels were decreased in both cases, but the cardiovascular risk reduction forecast reached 15% for the Mediterranean diet versus 9% for the low-fat diet. Through the above results after a 3-month Mediterranean diet intervention, there were significant changes on the serum lipid profile, decreasing possibly the risk of cardiovascular diseases (Vincent-Baudry et al. 2005).

Randomized Controlled Trials

In a further 3-month dietary intervention study, 772 individuals divided into 3 groups followed the Mediterranean diet (either with a high intake of olive oil or nuts) and a similar type of low-fat diet. The two types of diets based on Mediterranean dietary patterns were associated with a statistically significant improvement in serum lipid profile and glucose levels compared to the third group, in which it is worth noting that such intensive nutritional training had not been done. Therefore, a balanced diet based on Mediterranean dietary pattern and supplemented with olive oil or nuts may lead to a reduction in cardiovascular disease appearance owing to the positive effect of the above diet on significant parameters of serum lipids (Estruch et al. 2006).

372 subjects at a high cardiovascular risk (210 women and 162 men, aged 55–80 years) who were recruited into a large, multicenter, randomized, controlled, parallel-group clinical trial directed at testing the efficacy of the traditional Mediterranean diet on the primary prevention of coronary heart disease were assigned to a low-fat diet or 1 of the 2 traditional Mediterranean types of diets (traditional Mediterranean diet + virgin olive oil or traditional Mediterranean diet + nuts). After the 3-month intervention, there were significant changes, especially decreased oxidized LDL-C concentrations in the traditional Mediterranean diet + virgin olive oil group, while there were no changes on serum lipid profile in the low-fat diet group. Therefore, significant reductions in cellular lipid levels and LDL-C levels were observed in individuals at a high cardiovascular risk who improved their diet toward a traditional Mediterranean dietary pattern (Fito et al. 2007).

A randomized parallel controlled-feeding trial was conducted in 60 non-diabetics with mild abdominal obesity. After a two-week run-in with a diet high in saturated fatty acids, subjects were allocated a monounsaturated fat-rich diet, a Mediterranean diet, or a high saturated fat diet, for 8 weeks. The adherence to monounsaturated fat diet and the Mediterranean diet did not influence fasting insulin levels, but the high monounsaturated fat diet decreased TC and LDL-C relative to the high saturated fat diet and alongside the Mediterranean diet increased HDL-C levels, conversely reducing the TC/HDL-C ratio compared with the high monounsaturated fat diet. Consequently, replacing a high saturated fat diet with a high monounsaturated fat diet or a Mediterranean diet improved serum markers of lipids, with the Mediterranean diet being the most effective (Bos et al. 2010).

Taking into account the previous research that has identified several metabolites related to the Mediterranean dietary pattern, knowledge about longitudinal changes in metabolic biomarkers after a Mediterranean diet intervention is quite limited. A subsample of 48 firefighters cluster-randomized trial at Indianapolis fire stations was randomly selected for the metabolomics study at 12 months of follow-up, where Group 1 ($n = 24$) continued for further 6 months in a self-sustained Mediterranean diet intervention and Group 2 ($n = 24$), the control group at that time, began with an active Mediterranean diet intervention for 6 months. At the two times points, a total of 225 metabolites were examined. The Mediterranean diet intervention was associated with significant changes in biomarkers related to lipid metabolism, including decreased LDL-C, ApoB/ApoA1 ratio, remnant cholesterol, and VLDL-C and conversely greater HDL-C and better lipoprotein composition. In general, this intervention induces modest changes in adherence to the Mediterranean diet and consequently in metabolic biomarkers (Sotos-Prieto et al. 2020).

Comparative Study

The Seven Countries Study of 12,763 middle-aged men selected from 16 cohorts around the world underlined the protective effect of the Mediterranean dietary pattern against atherosclerosis that can be partially attributed to the decrease of blood pressure levels. A great part of the scientific community has recently associated the Mediterranean diet with improvements in blood lipid profile, reduced oxidation of lipids, and decreased risk of thrombosis (i.e., fibrinogen levels), all changes meaning improvement in endothelial function (Kromhout et al. 2018).

BioCycle Study

In the BioCycle Study (a prospective cohort study of menstrual cycle function) conducted between 2005 and 2007, 259 healthy premenopausal women, aged 18–44 years from western New York, were followed for 1 ($n = 9$) or 2 ($n = 250$) menstrual cycles, investigating in those whether Mediterranean diet adherence was associated with lower lipid peroxidation levels. Adherence to the Mediterranean dietary pattern has been correlated with a reduction in LPO levels and higher ascorbic acid concentrations, decreasing the risk of cardiovascular diseases and total mortality.

Crossover Design Studies

In a crossover design after a 4-week run-in period with a healthy diet, eligible candidates were randomized into three diet sequences, with a common background diet enriched with virgin olive oil, walnuts, or almonds, lasting 4 weeks each. As regards the results, there were significant changes in serum lipid profile and oxidation and inflammation markers. In 18 volunteers who completed the study, low-density lipoprotein cholesterol was decreased from baseline by 7.3%, 10.8%, and 13.4% after the virgin olive oil, walnut, and almond diets, respectively, and corresponding reduction results were observed about the TC and LDL-C/HDL-C ratio. However, there were no changes in other lipid fractions, oxidation analytes, or inflammation biomarkers. All the above findings in of hypercholesterolemic patients showed that the consumption of virgin olive oil, walnuts, or almonds that are basic components of the Mediterranean diet was extremely significant to improve specific parameters of serum lipids (Damasceno et al. 2011).

A randomized, controlled, crossover design compared the effect of a Mediterranean diet with three to four daily servings of dairy (MedDairy) and a low-fat (LF) control diet on significant biomarkers (including serum markers of lipids) associated with cardiovascular diseases. Forty-one volunteers aged $\geq$45 years and at risk of cardiovascular disease were randomly allocated to their first dietary intervention, either the MedDairy or low-fat diet, following each intervention for 8 weeks, with an 8-week washout period separated interventions. According to the main observations of this research, compared with the low-fat intervention, the MedDairy intervention resulted in a significantly lower morning SBP, lower morning DBP and clinic SBP, significantly greater HDL-C levels, lower TG concentrations, and also lower ratio of TC to HDL-C. Nevertheless there were no statistically significant effects on CRP, plasma glucose, and serum insulin. In general, the conclusion following a Mediterranean diet with additional dairy foods led to significant positive changes in various markers of cardiovascular diseases, mainly serum lipids (Wade et al. 2018).

Cross-Sectional Study

A cross-sectional analysis was conducted on the data from 1290 volunteers of the Aragon Workers Health Study cohort, examining the effect of a Mediterranean dietary pattern, high in vegetables, fruits, fish, white meat, nuts, and olive oil, and a Western diet, high in red meat and fast and refined food. Compared with the participants in the lowest quintile of Western-type adherence, those in the highest quintile had 4.6 mg/dl lower HDL-C, lower ApoA1 concentrations, and a higher risk of having reduced HDL-C. On the contrary volunteers with a high Mediterranean diet adherence had 3.3 mg/dl higher HDL-C levels and 0.43 times lower ratio of TGs to HDL. Thus, it was obvious that the Mediterranean diet adherence is positively associated with plasma lipids, while the adherence to Western dietary pattern was related to abnormal serum markers of lipids (Penalvo et al. 2015).

PREDIMED Sub-study

A sub-study of the PREDIMED (Prevention with Mediterranean Diet) trial in 1139 volunteers with a high cardiovascular risk also aimed to analyze the relationship of polyphenol intake from a Mediterranean diet, measured by polyphenol extraction, with circulating inflammatory biomarkers and cardiovascular risk factors in elderly people. The main findings of the present research indicated that participants in the highest tertile of changes in urinary polyphenol excretion were associated with lower plasma inflammatory biomarkers such as IL6, TNF-a and other parameters compared with those in the lowest tertile. In addition, systolic and diastolic blood pressure decreased, and conversely plasma HDL-C increased in parallel with increasing urinary polyphenol excretion. Therefore, high polyphenol intake from the Mediterranean dietary pattern was correlated not only with decreased inflammatory biomarkers but also improved markers of serum lipid profile (Medina-Remon et al. 2017).

Retrospective Observational Study

A retrospective observational study was performed in 31 children with dyslipidemia (aged 3–14 years) to investigate a possible association between nutritional habits or a general lifestyle based on Mediterranean dietary pattern and serum lipid levels (TC, LCL-C, HDL-C, TGs, ApoA1, ApoB, and LP(a)). From this research, although there was no statistically significant correlation between children's serum lipid levels and Mediterranean diet compliance, breakfast consumption, and consumption of fruits, vegetables, fresh juices, desserts, and sodas, some parameters of serum lipids differed significantly. More specifically, children with increased physical activity had lower triglyceride levels relative to those with lower physical activity. Also, children who consumed only one meal per day had increased levels of TC, LDL-C, ApoB, and LP(a), compared with those who consumed more than three meals per day. Another significant association was that children who were breastfed less than 6 months had significantly increased LDL-C levels relative to children who were breastfed more than 6 months. Therefore, there is a correlation of general healthy dietary habits with specific parameters of lipid profile (Lampropoulou et al. 2020) (Table 1).

Functional Foods of the Mediterranean Diet with a Beneficial Effect on Serum Markers of Lipids

In the context of the Mediterranean diet, specific functional foods proved to have physiological health benefits, decreasing the risk of various chronic diseases apart from their basic nutritional functions. Most constituents of some typically Mediterranean foods with functional value are associated with long-term mitigation of certain diseases, while there are also some perceived to increase short-term well-being. Furthermore, nutraceutical of these types of foods is an additional term that has been lately used to define foods that can be used as means to promote health and protect against diseases (Johnston 2009).

Table 1 Summary effects of the Mediterranean diet on serum markers of lipids

Study type/duration	Total of volunteers	Main findings	Study reference
Lyon Diet Heart Study: A randomized secondary prevention trial/46 months	605	Mediterranean dietary model compared with other recommended diets led to a positive effect on basic parameters of serum lipids, especially a decrease of TC and LDL-C and TGs and conversely an increase of HDL-C, reducing thus the risk of cardiovascular or other severe chronic diseases	De Lorgeril et al. 1999
Longitudinal study in Europe (HALE)/2 years	2339	No adherence of a low-risk lifestyle (characterized by Mediterranean dietary habits, moderate alcohol consumption, increased physical activity, and abstinence from smoking) was at greater risk of coronary heart disease and generally cardiovascular diseases, with the higher risk to be attributed to the adverse lipid profile	Knoops et al. 2004
Medi-RIVAGE study/ 3 months	2282	Mediterranean diet led to 9% lower TC, 19% lower LDL-C concentrations, and 32% lower oxidized LDL-C levels relative to a Westernized diet	Panagiotakos et al. 2004
ATTICA study/1 year	3042	Greater adherence to Mediterranean diet is associated with elevated total antioxidant capacity levels and low oxidized LDL-C concentration, which may explain the beneficial role of this diet on the cardiovascular system	Pitsavos et al. 2005
Medi-RIVAGE study/ 3 months	212	Mediterranean diet was concluded to have improved serum lipid profile and reduced body mass index and glucose levels, decreasing thus the risk of cardiovascular diseases	Vincent-Baudry et al. 2005
Randomized trial/1 year	772	Dietary types based on Mediterranean dietary patterns were associated with a statistically significant	Estruch et al. 2006

(continued)

Table 1 (continued)

Study type/duration	Total of volunteers	Main findings	Study reference
		improvement in serum lipid profile and glucose levels	
Seven Countries Study	12,763	Mediterranean diet was associated with improvements in blood lipid profile (especially in LDL-C and TGs), reducing oxidation of lipids and the risk of thrombosis	Dontas et al. 2007
Randomized controlled parallel-group clinical trial/ 3 months	372	Significant reductions in cellular lipid levels and LDL-C concentrations were observed in individuals at high cardiovascular risk who improved their diet toward a traditional Mediterranean dietary pattern	Fito et al. 2007
Randomized parallel controlled-feeding trial	60	Replacing a high saturated fat diet with a high monounsaturated fat diet or a Mediterranean diet improved serum markers of lipids, with the Mediterranean diet being the most effective to lipid profile (increasing HDL-C levels and reducing TC/ HDL-C ratio)	Bos et al. 2010
BioCycle study (a prospective cohort study of menstrual cycle function)/ 2 years	259	Mediterranean diet adherence has been correlated with a reduction in LPO levels and higher ascorbic acid concentrations, decreasing the risk of cardiovascular diseases and total mortality	Gaskins et al. 2010
Crossover design/4 weeks	18	Consumption of virgin olive oil, walnuts, or almonds that are basic components of the Mediterranean diet was extremely significant to improving specific parameters of serum lipids (reduced levels of LDL-C, TC, and LDL/HDL ratio)	Damasceno et al. 2011
Croatian study	1290	High Mediterranean diet adherence was positively associated with plasma lipids, especially 3.3 mg/dl higher HDL-C levels and 0.43 times	Penalvo et al. 2015

(continued)

Table 1 (continued)

Study type/duration	Total of volunteers	Main findings	Study reference
		lower ratio of TGs to HDL, while low Mediterranean diet adherence was related to 4.6 mg/dl lower HDL-C levels and lower ApoA1 concentrations	
PREDIMED study (a large, parallel-group, multicenter, randomized, controlled clinical trial)/4.8 years	1139	High polyphenol intake from the Mediterranean dietary pattern was correlated with decreased inflammatory biomarkers such as IL6, TNF-a, and other parameters and also improved markers of serum lipid profile (reduced systolic and diastolic blood pressure and HDL-C increased)	Medina-Remon et al. 2017
Randomized, controlled, crossover design/8 weeks	41	MedDairy intervention resulted in a significantly lower morning systolic blood pressure, lower morning DBP and clinic SBP, greater HDL-C levels, lower TG levels, lower ratio of TC to HDL-C, and no statistically significant effects on CRP, plasma glucose, and serum insulin	Wade et al. 2018
Pilot cluster-randomized trial/ 18 months	48	Mediterranean diet intervention was associated with significant changes in lipid biomarkers (decreased LDL-C, ApoB/ApoA1 ratio, remnant cholesterol, VLDL-C, while greater HDL-C and better lipoprotein composition)	Sotos-Prieto et al. 2020
Retrospective observational study/18 months	31	There was no correlation between children's serum lipids levels and Mediterranean diet compliance, breakfast consumption, consumption of fruits, etc., but children with higher physical activity had lower TG levels relative to those with lower exercise; children who consumed only one meal per day had higher TC, LDL-C, ApoB, and LP(a)	Lampropoulou et al. 2020

(continued)

Table 1 (continued)

Study type/duration	Total of volunteers	Main findings	Study reference
		levels compared with those who consumed more than three meals per day, and children who were breastfed less than 6 months had higher LDL-C levels relative to those who were breastfed more than 6 months	

Studies in human or non-human subjects indicate the importance of specific conventional or processed functional food intake within a Mediterranean dietary pattern, for the prevention of cardiovascular diseases; this effect is measured by specific biomarkers mainly serum lipids levels, as the abundance of bioactive compounds seems to have a positive effect on new or more functional goals of the human body, with a huge benefit for its health. Oleic acid, dietary fibers such as pectins, beta-glucans, and inulin; phytosterols; monounsaturated and polyunsaturated fatty acids; antioxidant vitamins and minerals; antioxidants; phytochemicals; and bioactive peptides are characterized as the basic functional components with protective effects against various pathological conditions of the host. The compliance with a diet based on Mediterranean dietary pattern characterized by a high consumption of several functional food categories or types, especially fruits and vegetables (wild greens, soy, pomegranate, olive oil, cranberries, garlic tomatoes, grapes, etc.), whole grains, nuts, fishes, red wine, herb drinks, and fortified foods (enriched with vitamins and minerals), may be significantly associated with the improvement of serum markers of lipids related to the human health status. Some of the typically functional foods from the Mediterranean diet with beneficial effects on serum lipid profile are listed in Table 2 (Koutelidakis and Dimou 2016).

Further Analyze the Significance of Specific Mediterranean Foods with Functional Value in Regulating the Serum Lipid Profile

Olive Oil

It is widely known that olive oil is a main fat component of the Mediterranean diet and contains an abundance of bioactive compounds, mainly oleic acid and polyphenols, with significant health benefits related to its positive effect on serum markers of lipids. In a randomized controlled trial, a basic finding was that a balanced diet based on Mediterranean dietary pattern with 30 grams (g) olive oil per day led to a statistically significant reduction in levels of SBP, TC, TGs, LDL-C, and VLDL-C. The above results indicated that olive oil may have a beneficial effect on specific parameters of lipid profile, reducing the risk of various chronic diseases (Atefi et al. 2018).

Table 2 Mediterranean functional foods with beneficial effects on serum markers of lipids (Koutelidakis and Dimou 2016)

Mediterranean functional foods	Bioactive compounds	Beneficial effect on serum lipid profile
Extra virgin olive oil	Polyphenols and oleic acid	Inhibition of LDL-C oxidation
Nuts	Tocopherols, omega-3 fatty acids, vitamin E	Lowering of blood cholesterol
Legumes	*Polyphenols and fibers*	Lowering of blood cholesterol
Fruits and vegetables	Fiber (pectin)	Lowering of blood cholesterol
Pomegranate	Polyphenols (anthocyanins)	Inhibition of LDL-C oxidation
Cranberries	Polyphenols	Inhibition of LDL-C Increase of HDL-C
Green leafy vegetables	Carotenoids	Inhibition of LDL-C oxidation
Citrus fruits and vegetables	Vitamin C	Inhibition of LDL-C oxidation
Low-fat dairy products (yogurt, etc.)	Probiotics	Inhibition of plasma cholesterol Inhibition of LDL-C
Fish oil	Omega-3 fatty acids, DHA, and EPA	Lowering of blood cholesterol Lowering of blood TGs
Fish	Omega-3 fatty acids	Inhibition of LDL-C oxidation Lowering of blood TGs Reduction of blood cholesterol
Whole grains (oats, barley)	Fiber and phytochemicals	Lowering of blood cholesterol Reduction of blood pressure
Tomatoes	Lycopene	Lowering blood cholesterol
Green tea	Tea polyphenols (catechins)	Inhibition of LDL-C oxidation
Green and black teas	Tea polyphenols	Reduction of blood pressure
Grapes and red wines	Grape polyphenols	Reduction of blood pressure
Onion and garlic	Quercetin	Reduction of blood pressure

Fruits and Vegetables

A main characteristic of the Mediterranean dietary pattern is the high consumption of fruits and vegetables. In the last years, there is an increased interest from the scientific community to investigate the positive interaction of these foods with a significant potential to decrease several chronic diseases, mainly cardiovascular diseases. Until now, the possible mechanism of the above correlation is that fruits and vegetables may protect LDL-C from oxidation (Aburto et al. 2013). Also the high content of them in

vitamins, minerals and micronutrients, beta-glucans, fibers (psyllium) that act together synergistically leads to inhibition of cholesterol synthesis and reduced LDL-C levels. Also in a randomized placebo-controlled double-blind clinical trial with a sample of 151 patients on hemodialysis, a main observation was the role of vitamin C supplementation contained in fruits with a view to reducing the CRP concentrations (Biniaz et al. 2014) and decreasing the LDL-C levels (Aguirre and May 2008).

Carotenoids (lycopene and beta-carotene) that are contained in carrots, tomatoes, and dark-green vegetables decrease oxidative stress in vivo. Moreover, healthy adults' intake of two cups per day of tomato juice for 2 weeks led to a reduction in plasma levels of CRP and TNF. In an additional group of patients with grade-1 hypertension, a high intake of tomato extract for approximately 1 month reduced systolic and diastolic blood in plasma and LDL-C concentrations. Also bioactive compounds, mainly polyphenols (tannins, lignins, and flavonoids), found generally in fruits and vegetables may have a beneficial effect on various biomarkers, including the serum lipid profile (Puglisi et al. 2008; Koutelidakis 2015).

Pomegranate

A typical example of food rich in polyphenols is pomegranate, a traditional Greek functional food belonging to the Mediterranean diet. The high content of pomegranate polyphenols may be related to the reduction in blood pressure and triglyceride concentrations and conversely an increase of high-density lipoprotein cholesterol (Shema-Didi et al. 2014). Another fruit rich in bioactive compounds with beneficial effects on serum lipid profile is cranberry. Particularly research data has proven that cranberry intake (either whole fruit or its juice) may be significantly associated with a decrease of LDL-C levels and conversely an increase of HDL-C concentrations, preventing cardiovascular diseases (Zikria et al. 2010).

Raisins

The scientific community elaborates on the significance of Mediterranean raisins, focusing especially on indigenous Greek varieties (e.g., Zante currants), widely known in food industry due to their high functional value attributed to their rich content in bioactive compounds (Papadaki et al. 2021). Particularly the above variety of raisins is a significant source of dietary fibers and polyphenols that may decrease cardiovascular disease risk by influencing lipoprotein and inflammation. The above functional food is included to the traditional Mediterranean diet, and its consumption based on study findings is related to reduced SBP, TC, and LDL-C levels and decreased TNF-a. Thus, research data indicates that simple lifestyle modifications such as adding raisins to a balanced diet may have beneficial effect on serum markers of lipids, lowering the risk of appearance of various chronic diseases (atherosclerosis, coronary heart disease, etc.) (Puglisi et al. 2008).

Wild Greens

Wild greens (fennel, radish) are among typical Mediterranean foods that belong to the category of vegetable with a high functional value. These foods may have a protective effect against atherosclerotic process and thus cardiovascular disease

development. This protective effect is due to the bioactive compounds of these foods, including phytochemicals and polyphenols such as sulforaphane, pectin, quercetin, etc. Possible mechanisms of their action include improving endothelial factors, thrombotic factors, and serum lipid profile (Lampropoulou et al. 2020).

Aromatic Plants and Essential Oils

The abundance of aromatic plants, such as oregano, mint, rosemary, sage, garlic, onion, etc., found in Mediterranean diet has been investigated in recent years for their possible health benefits owing to the bioactive components (oleuropein, resveratrol, quercetin, sulforaphane, anthocyanins, tannins, etc.,) of those functional foods. The possible effect of aromatic plants may be the result of either their polyphenolic extract or their essential oils. Essential oils and their constituents have been demonstrated to have a high antioxidant activity, protecting against LDL-C oxidation. Furthermore essential oils of several food components in the Mediterranean diet, of plant origin, are rich in phenolic compounds, lowering the concentrations of total cholesterol and triglycerides (Koutelidakis and Dimou 2016).

Fish and Fish Oil

One of the main foods that are included to the Mediterranean diet is fish, which is a major food source of long-chain n-3 polyunsaturated fatty acids (including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) that contribute to lowering blood pressure and decreasing also plasma triglyceride and low-density lipoprotein cholesterol levels (Mozaffarian 2008). Apart from fish, the intake of fish oil supplements inhibits hepatic triglyceride secretion and contributes to clearance of triglyceride in plasma, having thus a positive effect on serum markers of lipids (Ueeda et al. 2008).

Nuts and Walnuts

Apart from fish that was previously analyzed as a source of polyunsaturated fatty acids, there are other functional foods found in the Mediterranean diet with a high content of the above dietary nutrient. Particularly nuts (almonds, etc.) are traditional Mediterranean foods rich in polyunsaturated fats, having a possible beneficial effect on serum markers of lipids. Research data showed that an almond-based diet led to a reduction in TC and LDL-C while preserving HDL-C concentrations (Spiller et al. 1998). Also recent epidemiological studies provide an evidence of favorable effect of nut consumption on serum lipid profile, as the primary mechanism by which nuts prevent from various diseases (cardiovascular diseases, etc.) is through improvement of lipid and apolipoprotein profile (Altamimi et al. 2020).

Undoubtedly frequent consumption of nuts was correlated with improved serum lipid profile and lowered risk of cardiovascular diseases. In order to explore more the above association, researchers investigated the role of walnut intake on serum lipids and blood pressure. The main finding of this research was that the long-term consumption of walnuts inhibited the serum levels of TC and also decreased the LDL-C/HDL-C ratio due to the higher reduction of LDL-C than HDL-C, induced by this food (Sabate et al. 1993).

Mushrooms

Mushrooms have functional value and are included to the Mediterranean diet. Several studies in human and non-human subjects indicated that dietary intake of specific mushroom species contributed to the decrease of serum lipid levels. Particularly diets containing shiitake mushroom decrease serum lipids and serum lipophilic antioxidant capacity in rats (Yu et al. 2016). Also a research showed the lipid-lowering effects of oyster mushroom (*Pleurotus ostreatus*) in humans, as the treatment with oyster mushroom soup led to a significant decrease of oxidized LDL-C levels and a significant tendency in lowering TC concentrations (Schneider et al. 2011).

Dairy Products

Although a great part of researchers have supported the correlation of full-fat dairy product consumption with an increased risk of cardiovascular diseases, there is a scientific viewpoint that the intake of low-fat dairy products, which belong to "light category" (functional foods for specified nutritional uses), in parallel with a balanced diet based on Mediterranean dietary pattern, is proposed to consumers with a high risk of cardiovascular diseases, with a view to managing their body weight and improving their serum markers of lipids. More specifically, in a clinical study, an adolescent who consumed 2 liters of skim milk per day for 3 weeks appeared to have a reduction in levels of plasma cholesterol and LDL-C, relative to those who consumed full-cream milk or yogurt (Rossouw et al. 1981).

Another research showed that daily consumption of 200 ml of a functional yogurt may have a beneficial effect on serum markers of lipids associated with cardiovascular diseases (Agerbaek et al. 1995). As an extension of the above information, in general the daily consumption of dairy products (yogurt, fermented milk, etc.) with a high content of probiotics and other beneficial bioactive compounds in permitted quantities defined according to the Mediterranean diet may lead to an improved lipid profile with a positive impact on human health (Koutelidakis 2015).

Herb Drinks (Green Tea, Et Cetera)

In the nutritional recommendations based on the Mediterranean dietary pattern, it is proposed to replace soft drinks with other functional drinks such as herb drinks. In this category there is a wide variety of teas; mainly green tea is rich in catechins (especially epigallocatechin) that are extremely effective as lipid-lowering factors by inhibiting significant steps in the intestinal absorption of dietary fat, cholesterol, and other lipids. According to recent research findings, the biological role of tea on serum lipid profile is a main result of its antioxidant effect (attenuation of low-density lipoprotein oxidation) and of its possible effect on the expression of specific genes with the ability to regulate various metabolic pathways associated with various chronic diseases (Koutelidakis 2015).

Another research examined the effect of green tea on postprandial levels of plasma total antioxidant capacity (TAC), serum lipids, CRP, and glucose in patients with cardiovascular disease. The most significant observation was the increase in TAC at 1.5 and 3 h after breakfast with tea and the decrease in serum uric acid at 1.5 h after breakfast with tea. Thus, frequent consumption of tea may have a

beneficial effect on specific markers of lipids associated with human health (Koutelidakis et al. 2014).

Red Wine

According to the dietary recommendation based on the Mediterranean dietary pattern, there is a permitted daily amount of red wine consumption (especially 100 ml per day for women and 200 ml per day for men). A prospective randomized trial was performed from 2009 to 2011 in 108 patients with carotid atherosclerosis in order to investigate whether a daily glass of red wine associated with lifestyle changes improves the serum lipid profile of the above volunteers. After this dietary intervention, there was a significant reduction in low-density lipoprotein cholesterol levels and also a decrease of LDL-C/HDL-C ratio. Additional significant reductions were obvious in the concentrations of total cholesterol and triglycerides (Droste et al. 2013).

Application to Prognosis, Other Diseases, or Conditions

Applications to Prognosis

In this chapter the major blood plasma lipids (TC, TGs, VLDL-C, LDL-C, HDL-C, etc.) (Burdge and Calder 2015) have been reviewed which together show remarkable correlations with various chronic diseases. In particular dyslipidemias are disorders characterized by abnormal amounts of serum lipids (decrease of HDL-C, possible increase of TC, VLDL, and LDL levels) which are considered to be one of the most significant modifiable risk factors for cardiovascular diseases (Rizvi and Nagra 2014). Diagnosis, management, and treatment of dyslipidemias are of high importance with a view to preventing atherosclerosis and decreasing the incidence of cardiovascular diseases (coronary artery disease, etc.), a leading cause of mortality in adulthood. It is possible that serum lipid biomarkers may be used clinically in investigating prognosis in patients (Lampropoulou et al. 2020).

Applications to Other Diseases or Conditions

In this chapter we review an analytical platform for rapidly determining serum lipid status. Application of this methodology suggests that there is an aggravated serum lipid profile in patients with cardiovascular diseases (Rizvi and Nagra 2014). Several socioeconomic factors have been related to disorders of lipid levels in adults, such as increased screen time, reduced physical activity, lower socioeconomic status, shorter sleep duration, and unbalanced diet. The primary treatment of dyslipidemia in adults includes healthy lifestyle, increased physical activity, and balanced diet based on Mediterranean dietary pattern. More specifically Mediterranean dietary habits, such as low consumption of sugar-sweetened beverages, saturated fat, and high-calorie products and conversely an increased intake of complex carbohydrates and functional foods (fruits, vegetables, whole grains, olive oil, flaxseed, nuts, fishes, fortified

foods enriched with phytosterols, vitamins, minerals, and antioxidants) improve serum lipid levels, reducing the risk not only of cardiovascular diseases (Koutelidakis and Dimou 2016) but also of obesity, metabolic diseases, and diabetes (Lampropoulou et al. 2020) which in turn are associated with abnormal blood plasma lipid biomarkers (Franz et al. 2003; Dallongeville et al. 2007).

Mini-dictionary of Terms

- **Apolipoproteins**: Proteins that bind lipids to form lipoproteins, whose main function is to transport lipids.
- **Blood lipids**: Lipids in the blood, either free or bound to other molecules.
- **Cholesterol**: The main representative of sterols, free or esterified with a fatty acid, and alongside it is a basic component of cell membranes and a precursor molecule of many steroids in the body.
- **Chylomicrons**: Ultra-low-density lipoproteins that transport food lipids from the small intestine to the tissues.
- **HDL**: High-density lipoprotein that transports cholesterol from the tissues to the liver.
- **Intermediate-density lipoproteins VLDL**: Intermediated products of VLDL metabolism.
- **LDL**: Low-density lipoprotein that transports cholesterol from the liver to tissues.
- **Lipids**: Heterogeneous group of molecules that share the common property of hydrophobicity as they are insoluble in water, but soluble in fat solvents, having a great significance in the human organism (storage of energy, constituents of cell membrane, constituents of neural tissue, as an insulator and protective coating, and as vitamins).
- **Lipoproteins**: Spherical complexes of lipids and proteins that transport a great part of lipids and fat-soluble vitamins to and from tissues and also contain the core of hydrophobic lipids surrounded hydrophilic lipids and proteins.
- **Phospholipids**: A class of lipids whose molecule has a hydrophilic "head" containing a phosphate group and two hydrophobic "tails" derived from fatty acids, joined by a glycerol molecule.
- **TGs**: Glycerol esters with fatty acids and present in the blood to enable the bidirectional transference of adipose fat and blood glucose from the liver.
- **VLDL**: Very-low-density lipoprotein for transport of endogenously synthesized fat to tissues.

Key Facts

Key Facts of Dyslipidemia

- Dyslipidemia is one of the top five major risk factors leading to cardiovascular disorders.
- It is a disorder characterized by abnormal amounts of serum lipids.

- The main characteristics of dyslipidemia are high TC, TGs, and LDL-C and conversely low HLD-C.
- The prevalence of the above disorder in early age shows a large increase in recent years worldwide and increases further when various factors coexist, such as obesity, low physical activity, unbalanced diet, unhealthy lifestyle, etc.
- The rates of dyslipidemia and mortality from diseases associated with this disorder may be reduced through a high degree of adherence to the Mediterranean diet, as compliance with the recommendations of this dietary pattern can improve serum lipid profile, decreasing TC, TGs, and LDL-C and increasing HDL-C.

Summary Points

- Various biomarkers especially serum lipid profile (TC, LDL-C, VLDL-C, HDL-C, TGs, Apo, LPO, etc.) are influenced not only by genetic background of individuals but also environmental factors, mainly their nutritional habits.
- There is a large part of clinical and epidemiological studies about the effect of fiber, unsaturated fatty acids, and other nutritional habits based on Mediterranean dietary pattern on reduced levels of TC, LDL-C or VLDL-C, LPO, TGs, interleukins, TNF, or other inflammatory markers and conversely an increased HDL-C and ApoA1.
- A high Mediterranean diet adherence may have a positive effect on serum markers of lipids and health benefits, while low compliance with the above dietary model is possibly associated with negative health outcomes, owing to its negative changes on the lipid profile.
- Further research is needed to confirm the possible effect of specific foods, especially fruits and vegetables (almonds, wild greens, tomatoes, pomegranate), whole grains (psyllium, oat), nuts, flaxseed, olive oil, beverages (tea and aromatic plant extracts, red wine), and fortified foods (with phytochemicals, polyphenols, vitamins, minerals, etc.) included to the Mediterranean diet adherence on serum markers of lipids.
- In the context of Mediterranean diet, the consumption of conventional functional foods, super foods, and processed novel functional foods from the industry, rich in bioactive compounds, is of high importance to design nutritional policies and promote healthy nutritional habits, improving the serum lipid profile.

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Fecal Volatile Organic Compounds

17

What They Are and How They Can Be Used as Biomarkers in Diet and Nutrition

Anthony R. Pecoraro and Troy A. Markel

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Abstract

Fecal volatile organic compounds (VOCs) are a burgeoning field of study as a biomarker of both normal human physiology and disease states. The compendium of commensal bacteria that comprise the human intestinal microflora create unique signatures of compounds which – when released and analyzed ex vivo – represent markers of intestinal health, diet and nutritional exposure, and presence or absence of disease. While fecal VOC analysis as it relates to diet and nutrition

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is in its relative infancy, recent studies are beginning to demonstrate the value of this noninvasive biomarker as a proxy of colonic response to dietary components. In this chapter, we review the origin of fecal VOCs, how they are analyzed, and current research into VOCs as it relates to diet and nutrition.

Keywords

Volatile organic compounds · Gas chromatography-mass spectrometry · Electronic nose

Abbreviations

E-nose	Electronic nose
GC-MS	Gas chromatography-mass spectrometry
SCFA	Short-chain fatty acid
SPME	Solid-phase microextraction
VOC	Volatile organic compound

Introduction

Fecal volatile organic compounds (VOCs) are an ever-present and changing set of compounds released into feces via the metabolism of nutrients by intestinal bacteria. As it goes, changes to the intestinal microflora result in changes to the compounds released into feces, and, should those compounds be analyzed, unique chemical signatures of the underlying bacterial composition are uncovered. Short-chain fatty acids, alcohols, aldehydes, and a host of other chemical compounds can be extracted, isolated, and analyzed. Some of these compounds also have a unique dietary source that can be traced back via its interaction with known colonic flora. In this chapter, we will first elucidate what, as a general concept, fecal VOCs are and how they can be extracted and investigated and then discuss dietary patterns and nutritional differences that contribute to unique VOC signatures. Finally, we will discuss the potential for VOCs in the diagnosis of aberrant nutritional patterns and disease.

The Origins of Fecal Volatile Organic Compounds

The human microbiome is a reflection not only of the underlying health of the host but also a function of the nutritional components to which they are exposed. The bacteria utilize dietary components as fuel and produce a wide array of compounds as a result of intermediate metabolism. As defined in *International Encyclopedia of Public Health*, "Volatile organic compounds (VOCs) are organic chemicals that readily produce vapors at ambient temperatures and are therefore emitted as gases from certain solids or liquids" (Thurston 2017). VOCs on the whole have been extensively studied in non-biologic contexts, including in soil, in the paper industry – measured from toner in office copiers – and in the effects of perfumes on individuals

with asthma (Stotzky et al. 1976; Wolkoff et al. 1993; Dales and Raizenne 2004). VOCs can be extracted from any excreted substance, and other sources will be discussed in brief at the end of this chapter. Fecal VOCs are those chemicals created as a result of bacterial metabolism in the colon that are readily emitted in gaseous form from stool. Given that human feces are the product that serves as the closest proxy of end-metabolism in the colon, analysis of odoriferous compounds released into the stool has promise in the analysis of the human intestinal microflora when such bacteria cannot be directly sampled. Unfortunately, while the presence of these compounds in feces has the potential to serve as a noninvasive biomarker for disease and nutritional state, one detriment has been the sheer number of unique VOCs that can be identified from a single stool sample. In a study from the UK, 162 unique carbon-based VOCs were identified in a sample of 137 patients (Bond et al. 2019). Targeting those compounds of most significance can prove arduous without a large sample size. Much recent research has been devoted to identifying a particular VOC "signature" that can be attributed to particular human physiologic states; this research will be reviewed in the remainder of this chapter.

Analysis of VOCs

Many patients and healthcare providers insist that distinct gastrointestinal states result in an identifiable stool smell – *Clostridium difficile* infection, for example, has a unique malodorous smell. Until recently, researchers have been unable to scientifically quantify these peculiar observations. The current gold standard for VOC analysis is gas chromatography-mass spectrometry (GC-MS) (Arasaradnam et al. 2014). More specifically, GC-MS performed on the headspace (HS-GC-MS) captured by the processing of fecal samples has been optimized to account for low VOC density in stored stool samples (El Manouni El Hassani et al. 2020). While HS-GC-MS has demonstrated remarkable specificity in the detection of individual VOCs, this process is time-consuming and costly.

Using trained animals for olfactory detection as a less specific method of VOC analysis has been employed that has shown promise in discriminating between physiologic states. "Sniffer mice" have been shown to detect dietary variations by urine odor above a set dilutional threshold of 10^{-6} v/v (Sato et al. 2017). "Sniffer dogs" have shown a 99% sensitivity and 97% specificity in the detection of colon cancers (Shah et al. 2014). Outside of fecal analysis, dogs have demonstrated an ability to detect hypoglycemia in patients with type 1 diabetes as well as lung cancer – with a specificity of lung cancer detection of 86% (Biehl et al. 2019; Reeve et al. 2020). The use of animal detection of VOCs is binary – that is, presence or absence of a specific state. The need for specific training to new conditions makes this application potentially arduous when needing to control for many factors.

Other promising technologies include the use of electronic noses. E-noses utilize a sensor array and predictive analytics to broadly define VOC profiles of a given sample by individual sensor response intensity. These tools have been employed in food safety, breath research, and – more recently – the analysis of fecal samples

Table 1 Comparison of available VOC testing modalities

Analytical modality	Benefits	Downsides	Cost
Trained scent-detection animals	Disease discrimination Can detect diet variability	Training required Unproven model for previously unstudied disease states	++
Electronic nose	Sensor array offers broad scope of VOC detection Portable, quick, easy to operate Predictive analytics for discrimination of target condition	Inability to identify and characterize individual compounds VOC profile dependent on specificity of sensor array	+
Head space gas chromatography-mass spectrometry	Ability to specifically characterize particular VOCs VOCs can be preprocessed to optimize signal and control for noise	Cost-prohibitive for large-scale studies Time-consuming Cannot be performed in real time on fresh samples	+++

This table describes the three most common methods of analyzing and comparing VOC profiles, compared by cost, benefit, and disadvantage. Cost metrics as compared to other listed modalities are denoted by the symbol "+"

(Gasparri et al. 2018; Bosch et al. 2019; Bonah et al. 2020). The e-nose has been used to discriminate inflammatory bowel disease and diabetes in human excrement against healthy controls (Arasaradnam et al. 2011). Similarly, e-noses have demonstrated the ability to accurately discriminate between patients with and without colorectal cancer as well as advanced adenomas (de Boer et al. 2014). In nonhuman subjects, e-nose technology has exhibited accurate identification of *Mycobacterium tuberculosis* complex in stool analysis of wild boars (de Jesús Beleño-Sáenz et al. 2021). By utilizing a handheld VOC sensor, researchers are able to quickly and reliably measure a VOC array which, while less specific than GC-MS, affords the ability to perform numerous tests on a variety of samples. This technology has remarkable clinical potential, as gold standard testing modalities are often costly, time-consuming, and invasive for subjects. A review of the testing modalities described above can be found in Table 1.

Chemical Classification of VOCs

Recently, a compendium of identifiable VOCs in feces identified 381 distinct compounds, including methane from methanogenic bacteria and sulfides from sulfate-reducing bacteria (Amann et al. 2014). Alcohols, acids, ketones, esters, alkanes, alkenes, alicyclics, benzenoids, heterocyclics, sulfur-containing compounds, nitrogen-containing compounds, and chlorinated compounds have all been identified in human feces (Garner et al. 2007). Many of these groups of compounds can be directly attributed to either a particular dietary source or, more specifically, a particular type or species of colonic microflora. For example, short-chain fatty

acids (SCFAs) are particularly prominent among fecal VOCs because dietary carbohydrates serve as substrates for bacterial fermentation that produces SCFAs (Wong et al. 2006).

To add to the complexity of VOC analysis, VOC profiles from stool can vary widely between individuals. A study from 2009 identified 135 different VOCs, 22 of which were found in every subject and 34 of which were specific to individual subjects (De Preter et al. 2009). More recently, a study from 2013 identified 13 target compounds for analysis and found marked variability in their concentrations across subjects (Walton et al. 2013). In sum, while VOC analysis is complex, the sheer volume of data affords an excellent possibility to noninvasively unravel some of the mystery of the human microbiome.

Specific Dietary Components and Their Effect on VOC Profiles

Much attention is paid to diet and its relationship to systemic physiology. By using VOC analysis, we can begin to see how the substrates for bacterial metabolism supplied by diet can affect the microflora within. In 2020, researchers from Japan described the relationship between a high-fat diet and/or obese and diabetic subjects – a murine model was used – and excreted fecal VOCs. N-Alkanals, acetone, and phenol were noted to be elevated in obese/diabetic subjects and those who were fed a high-fat diet as opposed to a normally balanced diet. Interestingly, these metabolites also induced activity in a pro-inflammatory murine macrophage cell line, suggesting that a high-fat diet may be associated with a chronic, low-level inflammatory state. This is a particularly poignant observation, as the transition from low-fat to high-fat diets has been associated with the development of "Westernized" diseases such as type 2 diabetes (Wilson et al. 2020).

Additionally, researchers have investigated the role of dietary fiber on fecal VOC profiles. Dietary fiber has been shown to improve fasting blood glucose and hemoglobin A1C, a marker of long-term blood glycosylation (Post et al. 2012). In 2019, researchers from the Netherlands investigated the role of sugar beet pectin on the concentration of fecal SCFAs as measured by gas chromatography. Interestingly, SCFA was not significantly altered by dietary fiber supplementation, suggesting that while fiber may exert positive effects on systemic health, additional supplementation does not alter the metabolism of SCFAs in the colon.

In patients with iron-deficiency anemia, first-line treatment is often oral iron supplementation. Unfortunately, this treatment is frequently fraught with gastrointestinal side effects. Notably, fecal VOC analysis demonstrated changes that are typically associated with an inflammatory state consistent with reported symptoms. The concentration of several esters was found to be decreased after iron supplementation. Aldehydes on the other hand, which can be generated by lipid peroxidation as a measure of oxidative stress, were found to be elevated. While oxidative stress cannot be directly observed in the colon during therapy, identifying and monitoring the metabolic endpoints could help guide treatment wherein dietary supplementation is the mainstay (Ahmed et al. 2020).

Dietary Patterns and VOC Profiles

Starting in infancy, human fecal VOC profiles have been shown to be associated with dietary composition. In 2018 researchers demonstrated a significant difference in VOC profiles between preterm infants predominantly formula-fed and those predominantly breast milk-fed (El Manouni El Hassani et al. 2018). A similar study in 2020 demonstrated again that breast milk- and formula-fed infants differ in their fecal VOC profiles (Hosfield et al. 2020).

While – like most aspects of human physiology – there is much change in early life, studies have demonstrated that the same is true in adulthood, at least as it relates to intestinal microbial composition and fecal VOC profiles. Researchers in 2017 investigated the effect of dietary composition on the VOCs of patients with irritable bowel syndrome (IBS). Patients on a low FODMAP (fructans, galacto-oligosaccharides, lactose, fructose, and polyals) were compared to sham diet patients in their response on the IBS symptom severity scale. At the end of low-FODMAP treatment, nine unique VOCs were directly attributable to 31% of the response to dietary intervention (Rossi et al. 2018). The ability to predict response to dietary intervention affords clinicians the possibility of targeting therapy more precisely and achieving a more accurate prediction of treatment effectiveness.

In another study of 211 patients, fecal VOCs were compared across a variety of lifestyle factors, including diet, smoking status, and gender. The researchers found that those on a vegetarian diet had significantly different VOC profiles from those on a less restrictive diet (Bosch et al. 2019). While observational in nature, this study highlights another facet of the role dietary products – in this case the animal meat and fat missing from a vegetarian diet – play in the metabolism of the intestinal microbiota. More work is needed to determine the downstream effects of these differing VOC profiles, including whether such VOC arrays are associated with systemic or local inflammation.

VOCs from Other Sources

Urine, sweat, saliva, and breath have all been investigated with regard to excreted VOCs. In Poland, urine VOCs were analyzed to assess response after initiation of an oligosaccharide-enriched inulin supplement in patients with celiac disease on a gluten-free diet. Samples were analyzed using SPME and GC-MS. The concentration of benzaldehyde was noted to drop 36% on average with supplementation. This is potentially related to the drop in *Lactobacillus* concentration, which converts phenylalanine to benzaldehyde (Drabińska et al. 2019). Studies have shown that products of metabolism in the blood can be excreted in saliva and thereafter analyzed; salivary analysis, however, can be confounded by a number of other products including gastrointestinal reflux and gingival exudate (Broza et al. 2015). VOC profiles in the breath have shown considerable change after transition from a chow diet to a semi-purified diet in mice (Kistler et al. 2014). In all, VOCs from

excreted products are a proxy of the metabolism via the blood and/or colonic microflora and may reflect changes to a variety of physiologic states.

Applications and Further Study

Dietary intervention will likely play an ever-increasing role in systemic disease as more is understood about the composition and function of the intestinal microflora. For example, the "Western diet" has been associated with elevated levels of inflammation and intestinal dysbiosis, while vegetarian and "Mediterranean" diets have been shown to prevent dysbiosis and inflammation (Tomasello et al. 2016).

The ketogenic diet, a form of very low carbohydrate dieting that prioritizes dietary fats for fuel and encourages the endogenous production of ketone bodies as a primary energy source, has been used in the treatment of epilepsy for some time. Exposure to the ketogenic diet very rapidly alters the gut microbiota, promoting two bacterial species, *Akkermansia* and *Parabacteroides*. These bacteria increased colonic γ-glutamyl transpeptidase, which ultimately had the function of increasing the γ-aminobutyric acid (GABA)-to-glutamate ratio in the blood and brain, which could help minimize epileptogenic activity (Paoli et al. 2019). While VOC studies are limited in the analysis of the ketogenic diet, one such manuscript focused on VOCs from breath did demonstrate significant differences in some key VOCs between children with neurologic disease treated with and without ketogenic diet (Ruzsányi et al. 2018). More work is needed in the field of fecal VOC analysis as it may offer utility in predetermining response to dietary intervention for patients with seizure disorders who are candidates for the ketogenic diet.

Conclusion

Gut microflora has been studied extensively in recent decades as something of a key to unlocking the gastrointestinal tract's relationship to human health. Metabolic diseases such as obesity, type 2 diabetes, and nonalcoholic fatty liver disease have been linked to aberrant intestinal bacterial colonization (Fan and Pedersen 2021). In addition to metabolic dysregulation, intestinal dysbiosis has been linked to disorders of mental health, contributing to the "brain-gut-microbiota" axis (Dinan and Cryan 2017; Järbrink-Sehgal and Andreasson 2020). Dietary interventions have been posited to restore intestinal bacteria homeostasis. To sample stool directly is a somewhat arduous scientific process, particularly when used with the intention of monitoring the role of diet over time. However, analysis of fecal VOCs holds promise as a means of proxying not only bacteria themselves but also their relative metabolic activity and relationship to the health of the human host.

Much work remains in the area of fecal-derived volatile organic compounds. Studies described previously have primarily focused on *what* is produced and can be extracted and analyzed. There are two prevailing questions that remain to be answered: how the VOC profiles reflect changes in systemic physiology, and what

any given individual will respond to with regard to nutritional intervention. The electronic nose holds much potential in the longitudinal analysis and monitoring of fecal VOC response to intervention, while HS-GC-MS can be used to elucidate granular information regarding specific aerosolized compounds.

Application to Other Diseases

In this chapter, we reviewed the origin of fecal volatile organic compounds, the current standards for analysis, and applications to the analysis of diet and nutrition. Given that fecal VOCs are a proxy for intestinal microbiota and colonocyte metabolism, much work is currently being done to investigate the role of VOC analysis as a noninvasive diagnostic modality.

In preterm infants, VOCs have been used to detect such devastating gastrointestinal disorders as necrotizing enterocolitis (NEC). A recent multicenter study in the UK utilized solid-phase microextraction GC-MS to identify VOCs relevant to preterm infants who would develop NEC against age-matched controls. Three clusters of VOCs were directly associated with the development of NEC, one of which was associated with 1.6 times increase in the odds of developing NEC for a single standard deviation increase in concentration (Probert et al. 2020). Importantly, VOCs can be used to diagnose NEC up to 4 days before clinical diagnosis, which could have dramatic effects on the long-term prognosis of these patients (Wright et al. 2021).

Another potential cohort in which VOC analysis could prove useful is the application to inflammatory bowel disease (IBD). We reviewed above that VOCs could predict response to dietary intervention in patients with irritable bowel syndrome. In patients with IBD – i.e., patients with Crohn's disease or ulcerative colitis – VOC analysis as both a diagnostic modality and proxy for treatment response has shown potential. An analysis of 87 subjects, including 42 with Crohn's or ulcerative colitis, was conducted in 2013 and showed that patients with Crohn's had a significantly higher concentration of ester and alcohol derivatives of SCFAs and indole compared to others. Moreover, patients who were treated for IBD in this study had VOC profiles more like healthy controls.

Mini-Dictionary of Terms

- Volatile organic compound: carbon-based metabolite of natural or nonnatural origin that can be aerosolized from solid and liquid samples.
- Gas chromatography-mass spectrometry: multiphase analytical process of gaseous samples involving the separation and identification of target compounds by mass and charge.
- E-nose: VOC analyzer utilizing sensor array and predictive analytics to profile aerosolized compounds from a sample.

Key Facts of Volatile Organic Compounds

Volatile organic compounds (VOCs) are carbon-based molecules which are created as products of secondary metabolism by bacteria in the human colon.

VOCs can be extracted from waste products by head space gas chromatography-mass spectrometry or analyzed via electronic nose.

Certain VOC profiles have been linked to diet, both in preterm infants (formula vs. breast milk) and adults (vegetarian vs. nonvegetarian diet).

VOCs can also be used to predict response to dietary intervention for systemic illness, including irritable bowel syndrome and inflammatory bowel disease.

Summary Points

- Volatile organic compounds in stool serve as a proxy for both the intestinal microflora composition and nutritional sources of colonic metabolism.
- Head space gas chromatography-mass spectrometry can be utilized to identify and quantify concentrations of specific VOCs.
- Electronic noses can analyze the entire VOC profile of a given sample and use predictive analytics to identify specific features of the underlying specimen, including nutritional content and disease state.
- Particular dietary components are metabolized to produce common VOCs, including short-chain fatty acids, alcohols, alkanes, and sulfur-containing compounds.
- VOC profiles can be used to discriminate between disease states, as well as predict the response to dietary intervention in such gastrointestinal disorders as irritable bowel syndrome.

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Calprotectin as a Biological Indicator in Nutrition

18

Insulin Resistance and Beyond

Alberto Zamora, Ana Inés Méndez, and José-Manuel Fernández-Real

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Abstract

The inflammatory myeloid-related protein complex calprotectin, also known as MRP8/14, is a heterodimer comprised of two intracellular calcium-binding proteins, S100A8 (MRP8) and S100A9 (MRP14), predominantly expressed in activated human neutrophils, monocytes, and macrophages. Calprotectin is actively secreted during the stress response of phagocytes in association with acute and chronic inflammation. In this sense, elevated serum concentrations of calprotectin have been reported in multiple chronic inflammatory conditions, including rheumatoid arthritis and inflammatory bowel diseases. Calprotectin was also increased in subjects with insulin resistance and other chronic, low-grade inflammatory diseases such obesity and type 2 diabetes and its chronic complications, including cardiovascular disease. Concordantly, a decrease of S100A8/A9 has been observed after bariatric surgery-induced weight loss. Adherence to a Mediterranean diet was also associated with decreased calprotectin level. Little is known about the potential influence of lifestyle factors such as smoking or physical activity on circulating concentrations of calprotectin. Therapeutic strategies for blocking S100A9 and its activity are recently under development in chronic inflammatory diseases.

Keywords

Calprotectin · MRP8/14 · S100A8-S100A9 · Inflammation · Inflammatory conditions · Insulin resistance · Obesity · Type 2 diabetes · Cardiovascular disease · Diet · Lifestyle factors

Abbreviations

ACS	Acute coronary syndrome
BMI	Body mass index
CRP	C-reactive protein
CVD	Cardiovascular disease
FC	Fecal calprotectin
ICU	Intensive care unit
IFN-γ	Interferon-γ
IL-1	Interleukin-1
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
NADPH ox	NADPH oxidase
NF-κB	Nuclear factor *kappa B*
RAGEs	Receptor for advanced glycation end products
RYGB	Roux-en-Y gastric bypass
S100A8/A9 or MRP8/14 or calgranulin A/B	Calprotectin
T2DM	Type 2 diabetes mellitus
TNF-α	Tumor necrosis factor
UFAs	Unsaturated fatty acids
VAT	Visceral adipose tissue

Introduction

The inflammatory myeloid-related protein complex calprotectin, also known as S100A8/A9 or MRP8/14 or calgranulin A/B, is a heterodimer comprised of two intracellular calcium-binding proteins, S100A8 (MRP8) and S100A9 (MRP8), predominantly expressed in activated human neutrophils, monocytes, and macrophages (Striz and Trebichavsky 2004). The heterocomplex S100A8/A9 is secreted to the milieu in response to inflammation, participating in the transendothelial accumulation of monocytes at the site of inflammation (Manitz et al. 2003). It is considered as a marker for neutrophil activation, and it is secreted during the stress response of phagocytes. Indeed, calprotectin, as a component of the innate immune system associated with leukocyte adhesion, chemotaxis, and phagocytosis, appears to promote recruitment and infiltration of macrophages and polymorphonuclear cells into the inflammatory lesions (Halayko and Ghavami 2009). The inflammatory factors interleukin (IL)-1, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α are known to induce the expression and secretion of the S100A8/A9 heterodimer (Rammes et al. 1997; Xu and Geczy 2000). Not unexpectedly, the positive relationships between calprotectins and inflammatory parameters, such as blood neutrophil count and C-reactive protein (CRP), suggest that immune system activation modulates circulating calprotectin concentrations (Nijhuis et al. 2009; Oosterwijk et al. 2020; Peng et al. 2011). In fact, elevated serum concentrations of calprotectin have been reported in multiple chronic inflammatory conditions. Elevated circulating calprotectin levels have been reported in a variety of chronic inflammatory conditions including renal failure (Ehlermann et al. 2006), rheumatoid arthritis (Foell and Roth 2004), allograft rejection (Sudan et al. 2007), inflammatory bowel disease (Moein et al. 2017; Sipponen and Kolho 2010; Alibrahim et al. 2015; Ramió-Pujol et al. 2020; Molander et al. 2012; Laharie et al. 2011; Colombel et al. 2020), *H. pylori*-infected mucosa (Leach et al. 2008), and different lung diseases (Kotsiou et al. 2021) and at ICU admission predicting long-term mortality risk (Wirtz et al. 2020).

In the last decades, circulating calprotectin has also been found increased in subjects with insulin resistance, obesity, type 2 diabetes, and cardiovascular disease (Oosterwijk et al. 2020), the focus of this chapter.

First, we will briefly review the main mechanisms involved in calprotectin action.

Main Mechanisms of Calprotectin Action (Table 1 and Fig. 1)

Calprotectin interacts with heparin and heparan sulfate glycosaminoglycan, the receptor for advanced glycation end products (RAGEs), the scavenger receptor CD36, and the toll-like receptor 4, able to mediate detrimental effects caused by the activation of NF-κB via the RAGEs, inducing pro-inflammatory gene expression (Leclerc et al. 2009).

Calprotectin is also able to regulate many important processes in the body through the sequestration of zinc, leading to marked inhibition of matrix metalloproteinases. The latter are zinc-dependent enzymes important in angiogenesis and inflammation. The human S100A8/A9 can be considered as an antimicrobial

Table 1 Biology of calprotectin

Signal transduction is important for inflammatory signal cascades and control
Arachidonic acid is transferred to gp91phox of the NADPH complex, while S100A8 binds to p67phox and rac-2 of the NADPH oxidase complex leading to the oxidative burst
It is important in mediating inflammatory cascades in the vicinity of the plasma membrane via interaction with RAGE and TLR4
Activity within the intracellular cytoskeleton and extracellular matrix is dependent on calcium and zinc
Expression mediates the inflammatory and migratory potential of myeloid cells
Regulates the maturation of myeloid cells
Recruits myeloid-derived suppressor cells (MDSCs) promoting cancer growth via inflammatory pathways
It is differentially expressed in various cancers
Enhanced secretion of the pro-inflammatory factors IL-6, ICAM-1, VCAM-1, and MCP1

Adapted from Joseph Markowitz and Carson (2013)

agent due to the capability of the complex to bind to multiple metal ions (Brophy and Hayden 2012; Striz and Trebichavsky 2004).

Calprotectin seems also an important agent in eicosanoid metabolism. The calprotectins in general are considered to be the main fatty acid carriers of neutrophils, given their high binding affinity potential. In fact, calprotectin can bind unsaturated fatty acids such as α-linolenic acids, linoleic acid, and oleic acid in a calcium-dependent manner (Lin et al. 2016). The binding of arachidonic acid and oleic acid to calprotectin can cause the protein structural changes which could be beneficial to play a biological role in inflammation processes.

Calprotectin is a likely target for oxidation due to its co-localization with neutrophil-derived oxidants at sites of infection and inflammation. Indeed, in clinical samples from patients with asthma or inflammatory bowel disease, oxidation-sensitive cysteine and methionine residues were found to be oxidized to cysteine sulfinic acid and sulfonic acid, methionine sulfoxide, and dehydromethionine, respectively (Magon et al. 2015).

In addition to extracellular functions related to anti-infectious host defense mechanisms, calprotectin is also involved in the regulation of kinase activities, cytoskeletal rearrangement, differentiation, and cell migration, with recent attention being focused on the involvement of S100A8/A9 in cancer (Ehrchen et al. 2009; Striz and Trebichavsky 2004).

Fecal Calprotectin as a Marker of Inflammation

In the Intestine

Inflammatory bowel disease is a paradigmatic disease in which a chronic inflammation of the intestinal wall is observed, varying widely from patient to patient, and is thought to be due to a loss of homeostasis of the immune system in individuals with

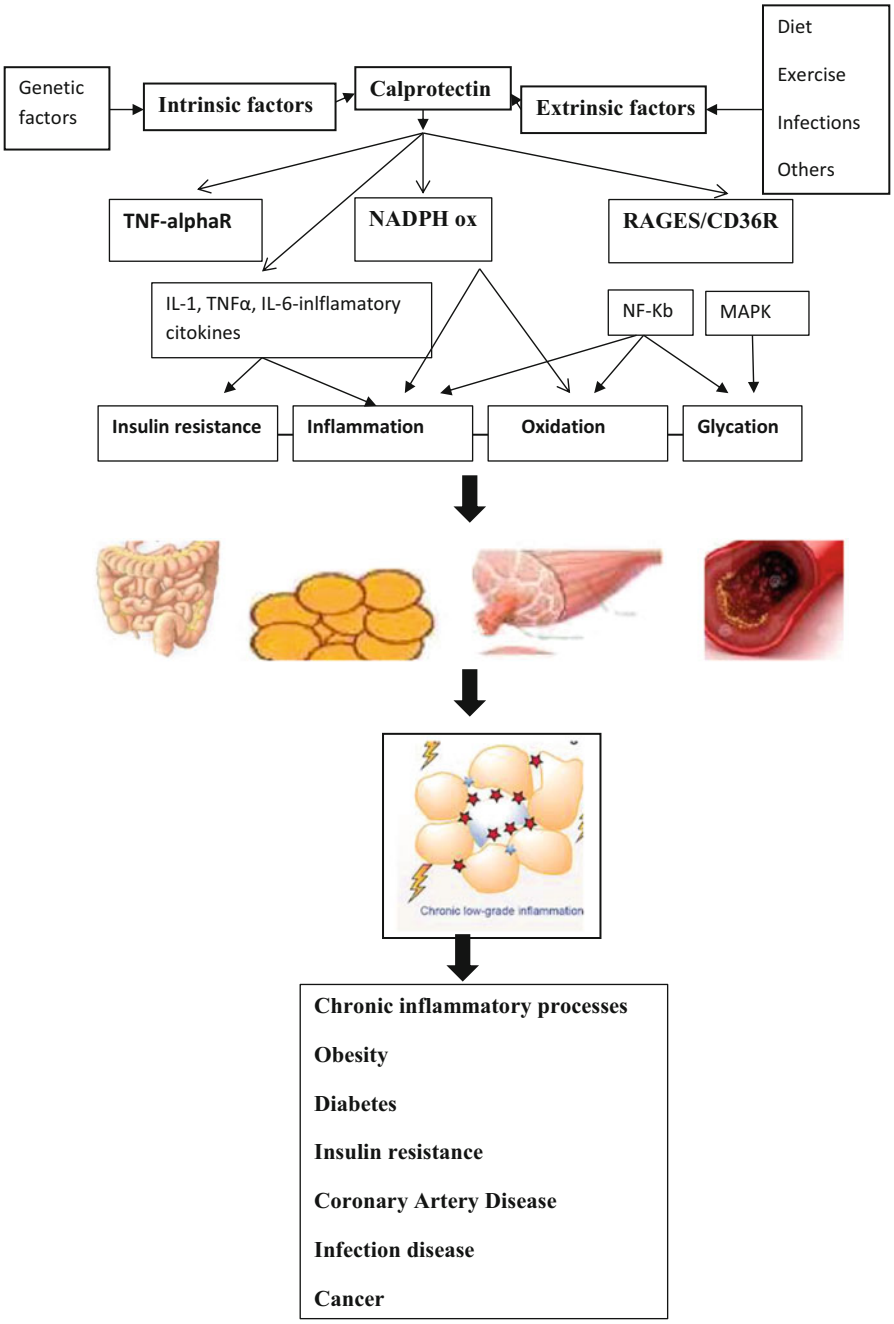

Fig. 1 Actions of calprotectin. TNF-alphaR, tumor necrosis factor alpha receptor; NADPH ox, NADPH oxidase; - RAGEs, advanced glycation end products; CD36R, receptor CD 36; NF-κB, nuclear factor *kappa B*; MAPK, mitogen-activated protein kinase

genetic predisposition. The prevalence of this disease has increased worldwide in recent years (Sýkora et al. 2018), highlighting the importance of a biomarker that is non-invasive, inexpensive, reliable, and with a good reproducibility.

Fecal calprotectin constitutes a biomarker highly correlated with clinical and histopathological activity. A recent study published in 2017 showed a sensitivity of 100% and a specificity of 80% for fecal calprotectin (at a cut-off of 160 μp/g) in the diagnosis of inflammatory bowel disease (Moein et al. 2017). Both urinary and fecal concentrations of calprotectin have been reported as early markers of disease activity (Ayling and Kok 2018). In fact, fecal calprotectin is also routinely used to detect subclinical inflammatory activity in asymptomatic patients (Sipponen and Kolho 2010). However, the intra-individual variability and the effect of certain drugs (non-steroidal anti-inflammatory drugs and proton pump inhibitors) may mask the diagnosis value of fecal calprotectin. Other diseases may result in a false positive result, mainly colorectal cancer, ankylosing spondylitis, and food allergies. The fecal amount of mucus or blood in the fecal samples should also be taken into account (Alibrahim et al. 2015). Furthermore, oral iron administration has been associated with increased levels of fecal calprotectin, suggesting increased gut inflammation (Gera and Sachdev 2002) even with the normal daily intake of iron (Kortman et al. 2015).

Fecal calprotectin allows the discrimination between functional and organic bowel processes, being a helpful tool in the differential diagnosis between inflammatory bowel disease and irritable bowel syndrome (Ramió-Pujol et al. 2020). In fact, fecal calprotectin can be used to select treatment strategies (Molander et al. 2012; Laharie et al. 2011). In this way, the CALM study showed that adjustment of therapy based on the combination of clinical symptoms and biomarkers led to better outcomes than symptoms-driven decisions (Colombel et al. 2020).

In pediatric patients with inflammatory bowel disease, a significant association between adherence to Mediterranean diet and a low level of fecal calprotectin has been described (Strisciuglio et al. 2020). In adults with colitis, the adherence to a Mediterranean diet was also associated with decreased calprotectin levels. Thus, the Mediterranean diet may have a role in modifying intestinal inflammation (Godny et al. 2020). In children with Crohn's disease under treatment with exclusive enteral nutrition, a reduction in fecal calprotectin concentrations has also been described (Logan et al. 2017; Gerasimidis et al. 2011).

Finally, elevated fecal calprotectin has been recently studied in patients with COVID-19 indicating intestinal inflammation (Mago et al. 2021) and suggesting that calprotectin represents an intriguing and promising biomarker for COVID-19 severity (Mahler et al. 2021).

In Chronic Low-Grade Inflammatory Disease

Obesity-Driven Insulin Resistance (Table 2)

It is well known that chronic subclinical inflammation is intrinsic to the metabolic syndrome (the clustering of central obesity and alterations of glucose and lipid

Table 2 Calprotectin and insulin resistance – diabetes

Author, Any	Findings
Mortensen 2009	Plasma calprotectin is a marker of obesity in individuals without type 2 diabetes
Peng et al. 2011	Diabetic patients with CAD had elevated plasma MRP8/14 levels which were also positively correlated with the severity of CAD and carotid IMT
Catalán et al. 2011	Increased levels of calprotectin in obesity and obesity-associated type 2 diabetes, its positive association with inflammation, as well as the higher expression levels in the stromovascular fraction cells in visceral adipose tissue
Ortega et al. 2012	Circulating and urinary concentrations of calprotectin are linked to chronic low-grade inflammation and insulin resistance beyond obesity
Pedersen et al. 2014	T2DM patients had higher concentrations of plasma calprotectin, which were associated with obesity, MetS status, autonomic neuropathy, PAD, and MI. However, plasma calprotectin was not an independent predictor of CVD, MI, autonomic neuropathy, or PAD
Calcaterra et al. 2018	Association between increased calprotectin and obesity also in children and suggest the potential utility of this biomarker in the monitoring of its metabolic complications

metabolism). Insulin resistance is central to the pathophysiology of these alterations, which runs together with the accumulation of fat and the presence of specific components (Nielsen et al. 2009; Shoelson et al. 2006). Obesity is associated with an increased risk of developing insulin resistance and type 2 diabetes mellitus (T2DM). In obese individuals, adipose tissue releases increased amounts of non-esterified fatty acids, glycerol, hormones, pro-inflammatory cytokines, and other factors that are involved in the development of insulin resistance (Kahn et al. 2006). It is now broadly accepted that obesity-associated low-grade chronic inflammation leads to the development of both insulin resistance and cardiovascular disease (Fernández-Real and Ricart 2003).

The inflammatory protein calprotectin has been described to be increased in subjects with obesity (Mortensen et al. 2009; Ortega et al. 2012; Catalán et al. 2011; Kunutsor et al. 2018).

Mortensen et al. measured plasma calprotectin and skeletal muscle S100A8 mRNA levels in a cohort consisting of 199 subjects divided into 4 groups depending on presence or absence of type 2 diabetes (T2D) and presence or absence of obesity. There was a significant interaction between obesity and T2D ($p = 0.012$). Plasma calprotectin was increased in obese relative to non-obese controls ($p < 0.0001$), whereas it did not differ between obese and non-obese patients with T2D ($p = 0.62$) (Mortensen et al. 2009).

Ortega et al. studied circulating calprotectin concentrations, other inflammatory markers, homeostasis model assessment of insulin resistance (HOMA-IR), and parameters of glucose and lipid metabolism were evaluated in 298 subjects (185 with normal (NGT) and 62 with impaired (IGT) glucose tolerance and 51 T2D subjects). Calprotectin was also evaluated in urine samples from 71 participants (50 NGT and 21 subjects with IGT). Insulin sensitivity (S(I), Minimal Model) was

determined in a subset of 156 subjects, and the effects of weight loss were investigated in an independent cohort of obese subjects ($n = 19$). Circulating calprotectin was significantly increased in IGT-T2D (independent of BMI) and positively associated with HOMA-IR, obesity measures, inflammatory markers, and parameters of glucose and lipid metabolism. Similar findings were reported for calprotectin concentrations in urine. Otherwise, weight loss led to decreased circulating calprotectin in parallel to fasting glucose and HOMA-IR (Ortega et al. 2012).

Catalan et al. studied in 53 subjects circulating concentrations and expression levels of calprotectin subunits (S100A8 and S100A9) in visceral adipose tissue (VAT), exploring its impact on insulin resistance and inflammation and the effect of weight loss. Circulating concentrations and VAT expression of S100A8/A9 complex were increased in normoglycemic and type 2 diabetic obese patients ($P < 0.01$) and associated with markers of inflammation ($P < 0.01$) (Catalán et al. 2011).

Kunutsor et al. analyzed 339 first CVD events. Serum calprotectin concentration was weakly correlated with several risk markers: positively with age, BMI, blood pressure, total cholesterol, nine triglycerides, and FPG and inversely with HDL-C. The strongest correlation was observed with hsCRP ($r = 0.42$) (Kunutsor et al. 2018).

Circulating calprotectin level correlated with the degree of adiposity both in healthy children and in children with excess body weight (Calcaterra et al. 2018; Grand et al. 2020).

Calcaterra et al. studied calprotectin in 131 children (11.7 ± 4.1 years) being higher in obese and overweight children than normal weight subjects ($p < 0.001$), with calprotectin in females being significantly higher than in males ($p = 0.04$). Increased calprotectin was related to pathological fasting blood glucose ($p < 0.001$) and insulin resistance ($p = 0.03$), while BMI ($p = 0.001$) and diastolic pressure ($p = 0.001$) are independent factors for increased calprotectin (Calcaterra et al. 2018).

A meta-analysis by Grand et al. that included data on 593 healthy children detected a positive correlation between BMI z-score and calprotectin, in girls (R: 0.48; $p < 0.001$) and boys (R: 0.39; $p < 0.001$). Multivariable analysis showed no significant associations with age, sample type (serum vs. plasma), or sex.

Circulating and urinary concentrations of calprotectin were also linked to chronic low-grade inflammation and insulin resistance beyond obesity (Ortega et al. 2012; Catalán et al. 2011).

Type 2 Diabetes-Driven Insulin Resistance

The relation circulating calprotectin to T2DM-driven insulin resistance is conflicting. While serum calprotectin was found to be significantly elevated in subjects with obesity compared to non-diabetic subjects without obesity, this difference was not found when comparing subjects with and without obesity but with T2DM (Mortensen et al. 2009). This finding was also observed in a Chinese study (Peng et al. 2011). However, higher serum levels of calprotectin have been found in persons with type 2 diabetes mellitus (T2DM) compared to healthy individuals (Pedersen et al. 2014).

Calprotectin and Macrovascular/Cardiovascular Disease

The number of experimental and clinical studies indicating that S100A8/A9 may favor the development of atherosclerosis is continuously increasing (Marinkovic et al. 2020; Miyamoto et al. 2008). McCormick et al. found that the arteries that did not develop atherosclerosis lacked the expression of S100A8 or S100A9. In fact, it is known that S100A9 may associate with lipid structures and may promote dystrophic calcification by altering the ability of phospholipid to bind calcium (McCormick et al. 2005). In this sense, in vivo studies in mice have shown that calprotectin promotes atherosclerosis (Croce et al. 2009). Interestingly, increased plasma concentrations of calprotectin were found to predict the risk of future cardiovascular events among healthy individuals (Healy et al. 2006). It is unclear whether calprotectin is simply a marker of systemic low-grade inflammation or plays a more specific role in the processes that promote atherosclerosis leading to CVD (Kruzliak et al. 2014). The usefulness of calprotectin as a CVD predictor is, however, not straightforward given the strong association of circulating calprotectin with smoking, a well-known direct cause of vascular inflammation.

However, it has been proposed that there is a log-linear association of calprotectin concentration with risk of CVD, which may be partly dependent on hsCRP. Adding calprotectin to conventional risk factors improves CVD risk assessment using measures of reclassification and −2 log likelihood (Kunutsor et al. 2018). Serum calprotectin has also been shown to be associated with cardiovascular disease in T2DM (Berezin 2016; Oosterwijk et al. 2020).

In addition, in the acute phase, circulating calprotectin was found to be an early and sensitive marker of acute coronary syndrome (Altwegg et al. 2007). The levels of plasma calprotectin appear to increase earlier than other markers of myocardial necrosis (myoglobin, creatine kinase-MB, and troponin), and high levels are associated with an increased risk of recurrent cardiovascular events (Morrow et al. 2008).

Further development regarding the possible predictive value of calprotectin to choose better-tailored strategy in the diagnosis of cardiovascular events is thus needed.

Calprotectin and Microvascular Disease

As calprotectin is positively associated with HbA1c, chronically increased levels of glucose or glycation products may modulate the impact of high calprotectin levels on chronic microvascular diabetic complications (Tabur et al. 2015). In fact, elevated calprotectin levels have been reported to predict microvascular alterations (Burkhardt et al. 2009) and to be associated with microalbuminuria (Schmaderer et al. 2014) in patients with T2DM. Levels of plasma calprotectin were also increased in diabetic peripheral neuropathy (Tabur et al. 2015).

In another study, plasma calprotectin levels correlated with BMI, fasting triglycerides, HDL cholesterol (inversely), hsCRP, insulin and C-peptide levels, as well as HOMA-IR in T2DM patients. Increased levels of plasma calprotectin were also found in T2DM patients with autonomic neuropathy, peripheral artery disease, and myocardial ischemia compared with patients without these conditions (Pedersen et al. 2014).

Puzzlingly, serum levels of calprotectin and calprotectin expression in monocytes have been found to be increased in type 1 diabetes patients and suggested to be involved in the pathogenesis of type 1 diabetes (Nyalwidhe et al. 2017).

Therapeutic Opportunities

Effects of Lifestyle and Diet on Levels of Calprotectin

Given the well-known increase of fecal calprotectin in inflammatory bowel disease and in metabolic disease, any intervention targeting intestinal inflammation through diet, as described above with a Mediterranean diet, is expected to decrease systemic calprotectin levels.

Weight reduction per se alters the balance of systemic inflammatory activity: the expression of different pro-inflammatory factors is decreased, while the levels of several anti-inflammatory molecules are increased (Fernández-Real and Ricart 2003; Calder et al. 2011). As expected, decreases of circulating S100A8/A9 and CRP are consistently observed after diet-induced weight loss (Catalán et al. 2011; Chen et al. 2009; Nijhuis et al. 2009) and after bariatric surgery-induced weight loss in T2DM patients in parallel to interleukin 6 (Lylloff et al. 2017; Miller et al. 2011; Netto et al. 2015; Nijhuis et al. 2009). In fact, the higher the S100A8/A9 and IL-6 gene expression, the higher the probability of persistent diabetes status post-surgically (Lylloff et al. 2017). Bariatric surgery also reduced the levels of sRAGE, a soluble form of the putative receptor of calprotectin. The elevated gene expression levels of the calprotectin subunits S100A8 and S100A9 in adipose tissue from subjects with obesity were related to monocyte macrophage content linked to raised TNF-α expression (Catalán et al. 2011).

Relatively, little is known about the potential influence of nutrition on circulating concentrations of calprotectin. In one study, plasma calprotectin was found to be independently associated with intake of mono- and disaccharides (Oosterwijk et al. 2020). In fact, the ingestion of modern Western food is considered to be pro-inflammatory and to be associated with hyperinsulinemia, increased intestinal permeability, and chronic low-grade inflammation (Ruiz-Núñez et al. 2013; Calder et al. 2011). The consumption of dietary monosaccharides fuels inflammatory processes in humans (Della Corte et al. 2018), and diets with decreased carbohydrate content are known to reduce systemic inflammation in patients with T2DM (Ohlsson et al. 2017). However, some food components in the Okinawan-based Nordic diet with decreased carbohydrate and increased fat and protein contents also triggered an inflammatory response with increased plasma calprotectin and zonulin release, a marker protein which reflects impaired intestinal barrier and increased intestinal permeability (Ohlsson et al. 2017).

Little is known about the potential influence of other lifestyle factors such as physical activity on circulating concentrations of calprotectin. One study showed that serum calprotectin levels were log-linearly correlated with smoking status (Oosterwijk et al. 2020), so smoking cessation is expected to decrease circulating calprotectin.

Future Prospects in the Potential Impact of Calprotectin Modulation

The blocking of S100A8/A9 may represent a modality to treat atherosclerosis via the downregulation of inflammatory pathways (Ehlermann et al. 2006). In fact, different therapeutic strategies for blocking S100A9 and its activity are under development in several inflammatory diseases (Markowitz and Carson 2013). Short-term anti-S100A9 blockade during the early inflammatory phase post-myocardial infarction can effectively mitigate post-ischemic myocardial damage, while long-term blockade may induce undesired side effects and offset the favorable consequences of the short-term treatment. Therefore, the search of an appropriate therapeutic window to achieve optimal effects of S100A9 blockade will be a hot topic with promising research prospects in the future (Cai et al. 2020; Pruenster et al. 2016).

In addition to the modulation of calprotectin using monoclonal antibodies, microRNA-24 could also constitute a therapeutic agent and an interesting candidate given its capacity to target calprotectin subunit S100A8 (Guo et al. 2012). These ideas, however, still need to be verified in large clinical trials, and different cut-off values for plasmatic levels of calprotectin should be established for personalized medicine in cardiometabolic diseases to reveal the true diagnostic, prognostic, and therapeutic potential of calprotectin.

Conclusions

Calprotectin is a major protein involved in a wide range of metabolic and cancer diseases. Calprotectin could also serve as an important prognostic factor and therapeutic target for cardiovascular and cardiometabolic diseases on the basis of their underlying chronic low-grade inflammation. Calprotectin may represent a useful marker in predicting the course of insulin resistance, obesity, and T2DM. Therapeutic strategies for blocking S100A9 and its activity are recently under development in different prevalent diseases, so calprotectin has potentially many more lessons to teach us in the future (Tables 3 and 4).

Mini-Dictionary of Terms

Calprotectin. Inflammatory myeloid-related protein complex also known as S100A8/A9, MRP8/14, or calgranulin A/B, which is a heterodimer comprised of two intracellular calcium-binding proteins, S100A8 (MRP8) and S100A9 (MRP8).

Insulin Resistance. When higher circulating insulin levels are necessary to achieve the integrated glucose-lowering response described above, a subject is considered insulin resistant. A variety of clinical entities – prediabetes, lipodystrophy, polycystic ovarian syndrome, and nonalcoholic fatty liver disease – are accompanied by increased fasting plasma insulin concentrations. This increased workload for the endocrine pancreas, and consequent β-cell decompensation, is a major mechanism for the development of overt T2DM.

Table 3 Clinical conditions associated with elevated calprotectin

Clinical conditions	Reference
Inflammatory bowel disease	Moein et al. 2017; Sipponen 2010; Alibrahim 2015; Sara Ramió 2020; Molander et al. 2012, Laharie 2011; Colombel 2018
Renal failure	Ehlermann 2006
Rheumatoid arthritis	Foell 2004
Lung diseases	Kotsiou 2021
H. pylori-infected mucosa	Leach 2008
Allograft rejections	Sudan 2007
Cancer	Ehrchen 2009; Striz and Trebichavsky 2004
Intrinsic acute kidney injury	Ehlermann 2006
Obesity	Mortensen 2009; Ortega 2012; Catalán et al. 2011; Calcaterra 2018; Grand 2020
Insulin resistance and metabolic syndrome	Kunutsor 2018; Ortega 2012; Catalán et al. 2011; Calcaterra 2018
Type 2 diabetes mellitus	Mortensen 2009; Peng et al. 2011; Pedersen 2014; Ortega 2012; Tabur 2015
Type 1 diabetes mellitus	Bouma 2004
Microvascular diabetes complications	Pedersen 2014; Burkhardt 2009; Tabur 2015; Schmaderer 2014
Atherosclerotic artery disease	Berezin 2016; Oosterwijk et al. 2020; Marinkovic 2020; Miyamoto 2007; McCormick 2005; Croce 2009; Healy 2006; Oosterwijk et al. 2020; Kunutsor 2018
Acute coronary syndrome	Altwegg 2007; Morrow 2008
Sepsis	Wirtz 2020
Severity of COVID-19 infection	Mago 2021; Mahler 2021

Matrix Metalloproteinases (MMPs). They are a family of zinc-dependent extracellular matrix (ECM) remodeling endopeptidases that have the capacity to degrade almost every component of the ECM. The degradation of the ECM is of great importance, since it is related to embryonic development and angiogenesis. It is also involved in cell repair and the remodeling of tissues.

Mediterranean-Style Diet. High monounsaturated/saturated fat ratio (use of olive oil as the main cooking ingredient and/or consumption of other traditional foods high in monounsaturated fats such as tree nuts) and a high intake of plant-based foods, including fruits, vegetables, and legumes. Additional components included low to moderate red wine consumption; high consumption of whole grains and cereals; low consumption of meat and meat products and increased consumption of fish; and moderate consumption of milk and dairy products. The intervention could be dietary advice, provision of relevant foods, or both.

***Receptor for Advanced Glycation End Products* (RAGE)**. It is a multi-ligand receptor considered as a key mediator of several physiological (e.g., tissue differentiation and regeneration/repair and resolution of inflammation) and pathological (e.g., inflammation, diabetes, cardiovascular diseases, neurodegeneration, and cancer) processes through the activation of multiple cellular signaling cascades.

Table 4 Impact of diet on calprotectin levels

Diet type	Calprotectin levels
Hypocaloric diet-weight loss	
Mediterranean diet	
Unsaturated fatty acids	
Enteral nutrition	
Western diet	
Okinawan-based Nordic diet	
Dietary sugar consumption	
Oral iron excess	

Key Facts of Calprotectin as a Biological Indicator in Nutrition: Insulin Resistance and Beyond

Physiology and functions of the calprotectin
- Fecal calprotectin is association with intestinal inflammation
- Calprotectin and its association with obesity
- Calprotectin and its association with insulin resistance and type 2 diabetes
- Calprotectin as a predictive marker of chronic complications of diabetes
- Calprotectin and cardiovascular disease
- Effect of lifestyle and diet on levels of calprotectin
- Applications of calprotectin to prognosis of other diseases or conditions
- Future prospects in the modulation of calprotectin

Summary Points

- Calprotectin is expressed in activated human neutrophils, monocytes, and macrophages.

- Calprotectin is actively secreted during the stress response and is associated with inflammation.
- Elevated serum concentrations of calprotectin have been reported in multiple chronic inflammatory conditions.
- Fecal calprotectin is highly correlated with clinical and histopathological activity of inflammatory bowel disease.
- Calprotectin is increased in subjects with insulin resistance, obesity, type 2 diabetes, and cardiovascular disease.
- Potential influence of lifestyle factors, such as smoking, physical activity, and nutrition, on circulating concentrations of calprotectin.
- Therapeutic strategies for blocking calprotectin and its activity are recently under development in inflammatory diseases.

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Omega-6 Fatty Acids

19

Biomarkers for Diet and Chronic Diseases

Xin Huang and Jie V. Zhao

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Abstract

Omega-6 fatty acids are involved in a series of biological processes including inflammation, immune function, and carcinogenesis. Omega-6 fatty acids are closely related to multiple chronic diseases, such as cardiovascular disease, diabetes, asthma, autoimmune diseases, and cancer, however, the evidence is controversial. Omega-6 fatty acids are also responsive to dietary intake and therefore serve as biomarkers for dietary intake. This chapter provides a narrative review of omega-6 fatty acids as biomarkers for major chronic diseases, summarizes the usefulness and limitations of omega-6 fatty acids as biomarkers in epidemiological research and clinical applications, with implicatoins for using omega-6 fatty acids as biomarkers for disease prediction, dietary interventions and primary health care.

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Keywords

Omega-6 fatty acids · Diet · Cardiovascular disease · Diabetes · Asthma · Autoimmune disease · Cancer

Abbreviations

5-HETE	5 hydroxyeicosatetraenoic
AA	Arachidonic acid
CHD	Coronary heart disease
CVD	Cardiovascular disease
D5D	Delta-5-desaturase
D6D	Delta-6-desaturase
DGLA	Dihomo-γ-linolenic acid
GLA	γ-linolenic acid
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LA	Linoleic acid
LT	Leukotrienes
LXA4	Lipoxin A4
MI	Myocardial infarction
MS	Multiple sclerosis
PUFAs	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor-α

Introduction

Omega-6 fatty acids (also known as n-6 fatty acids or ω-6 fatty acids) are a type of polyunsaturated fatty acids (PUFAs), where the final carbon-carbon double bond at the farthest end of the carboxyl group in the molecule is located on the sixth carbon atom counting from the methyl end (n-6). Linoleic acid (LA) and arachidonic acid (AA) are the most common omega-6 in diet, mainly derived from vegetable oils, cereals, red meat, poultry and so on. LA (18:2, n-6) is a precursor of AA (20:4, n-6), which can be converted into AA through γ-linolenic acid (GLA, 18:3, n-6) and dihomo-γ-linolenic acid (DGLA, 20:3, n-6) by desaturase and elongase enzymes (Fig. 1).

Omega-6 fatty acids are responsive to dietary intake, which can be used as biomarkers for diet. In addition, accumulating epidemiological studies suggest that omega-6 fatty acids are involved in the pathogenesis of multiple chronic diseases, such as cardiovascular disease, diabetes, asthma, autoimmune disease, cancer, etc., with the potential of being applied as biomarkers for these diseases. In this chapter, we provide a narrative review of omega-6 fatty acids as biomarkers for dietary

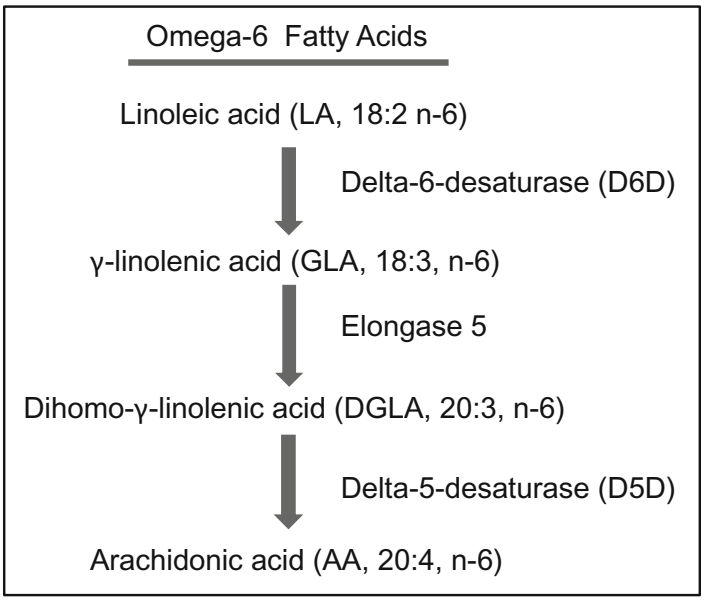

Fig. 1 Pathway of omega-6 fatty acids metabolism

intake and major chronic diseases, and summarize the potential and limitations of using omega-6 fatty acids as biomarkers in epidemiological research and clinical applications.

Omega-6 Fatty Acids as Biomarkers for Dietary Intake

Nutrition and diet are modifiable risk factors with the potential to prevent chronic diseases. In order to clarify the relationship between diet and diseases, dietary assessment has become a key issue. Most studies used the Food Frequency Questionnaires (FFQ) or 24-hour dietary recall as instruments to assess habitual dietary intake (Reigada et al. 2021). Although they are convenient to use in large epidemiological studies, they might be open to recall bias, for example, arising from incorrect estimation of portion sizes, and misclassification of food components (Kristal et al. 2005; Picó et al. 2019). Fat is often one of the most underestimated items in various dietary self-report surveys (Subar et al. 2003; Subar et al. 2001). To overcome the limitations, endogenous omega-6 fatty acids, responsive to dietary fat intake, can be used as biomarkers for dietary intake. In an intervention study conducted by Hodson et al. (2014), LA abundance was measured in participants during a intervention with diet enriched with omega-6 fatty acids; the percentage of total fatty acids in each lipid fraction (Plasma cholesterol ester, plasma phospholipid, plasma triglyceride, erythrocyte phospholipid, and buccal cell phospholipid) increased with LA during the first 2 weeks of the omega-6 diet period, suggesting

that changes in LA content can be used as a marker of dietary change. Baylin and Campos (2006) summarized the results of studies quantifying dietary compliance using fatty acids from plasma fractions and found a dose-response relationship with serum cholesterol ester LA following a gradual increase in LA intake. It is necessary to explore omega-6 biomarkers as alternative options for measuring dietary intake in epidemiological and clinical studies.

Assessment of omega-6 fatty acid biomarkers is readily accessible and diverse, using a variety of blood fractions, including total blood, plasma, serum, erythrocytes, as well as tissue sampling from adipose tissues. In most studies, blood fractions are more commonly used for biomarker measurements because they are easier to obtain and measure, compared to adipose tissues. Plasma and serum levels are considered to reflect short-term intake over a few days (Katan et al. 1997), while erythrocytes and adipose tissues are a better long-term marker due to the long half-life (Albert et al. 2002; Smedman et al. 1999) for example, 6 months to 2 years for LA in adipose tissue (Beynen et al. 1980; Hodson et al. 2014; Strawford et al. 2004). Experimental studies have also shown that adipose tissue can reflect dietary compliance within 5 years (Baylin and Campos 2006; Dayton et al. 1967). Total blood, plasma, serum, and erythrocytes all proved to be reliable in accessing fatty acid intake (Baylin et al. 2005; Hodson et al. 2014). The choice of measurement should take into account the cost of sample collection and preparation, as well as the objective of assessment.

Omega-6 Fatty Acids as Biomarkers for Cardiovascular Disease

Omega-6 fatty acids have long been believed to be related to cardiovascular diseases, and thus a potential biomarker for cardiovascular disease, while the evidence from different study designs is inconsistent. In case-control studies, serum and erythrocyte LA were lower in patients with coronary artery disease compared with controls (Block et al. 2008; Song et al. 2014). In contrast, serum AA were higher in patients than in healthy controls (Song et al. 2014). A meta-analysis of observational studies conducted by Harris et al. (2007) also showed that lower blood/tissue levels of LA were associated with an increased risk of CHD, while AA levels in adipose tissue were positively associated with CHD events. Marklund et al. (2019) conducted an individual-level meta-analysis in a global consortium of 30 prospective observational studies from 13 countries and found that higher in vivo circulating or tissue levels of LA were associated with lower risk of total CVD, cardiovascular mortality, and ischemic stroke, whereas AA was only weakly negatively associated with lower risk of total CVD. However, these observed beneficial associations have not been corroborated in randomized controlled trials (RCTs) and Mendelian randomization. Specifically, a recent systematic review and meta-analysis including 19 RCTs showed no clear benefit of omega-6 fatty acids on cardiovascular disease events and mortality (Hooper et al. 2018). Using Mendelian randomization to minimize confounding (Fig. 2), Zhao and Schooling (2019a) showed that genetically predicted endogenous LA

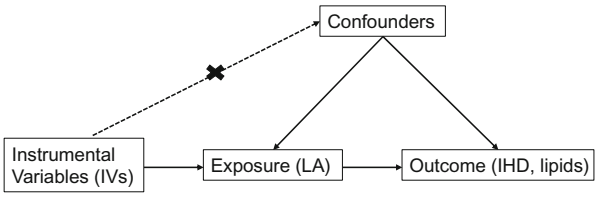

Fig. 2 Diagram of MR study

Table 1 Genetically predicted LA with IHD and lipids using Mendelian randomization

Exposure	Outcome	OR/ Beta	95% CI	P value
Linoleic acid (% in total fatty acids)	Ischemic heart disease (IHD)	1.01	0.95 to 1.08	0.71
	LDL cholesterol	− 0.032	− 0.037 to − 0.027	<0.001
	HDL cholesterol	− 0.026	− 0.031 to − 0.021	<0.001
	Total cholesterol	− 0.030	−0.035 to − 0.026	<0.001

From: Effect of linoleic acid on ischemic heart disease and its risk factors: a Mendelian randomization study

levels were inversely associated with lipids, but the association with CHD events was not significant (Table 1), which showed consistency with evidence from meta-analysis of RCTs.

Omega-6 Fatty Acids as Biomarkers for Diabetes

In addition to cardiovascular disease, omega-6 fatty acids are also relevant to diabetes. A systematic review and meta-analysis including 102 RCTs showed that compared to carbohydrate, saturated fatty acids and monounsaturated fatty acids, dietary polyunsaturated fatty acids (mainly LA) have beneficial effects on diabetes (Imamura et al. 2016). Wu et al. (2017) summarized the association of LA and LA biomarkers with incidence of T2D in 20 prospective cohort studies with a total of 39,740 participants, and showed that higher LA was associated with a lower risk of T2D, while the association was not observed for AA. In nested case-cohort studies from the European Prospective Investigation into Cancer (EPIC) cohort (15,919 participants), plasma LA was also inversely related to T2D, independent of AA (Forouhi et al. 2016).

Despite, Weir et al. (2020) proposed a new hypothesis that omega-6 fatty acids are more likely to be markers of hyperinsulinemia rather than protective or risk factors for T2D. Insulin may be the main driver of these associations by the mechanism that Insulin can stimulate D6D, leading to the conversion of LA to GLA, and then to DGLA. While stimulating D6D, increased long-chain PUFA will

inhibit D5D activity, resulting in reduced conversion of DGLA to AA (Arbo et al. 2011; Vessby et al. 2002). Consistently, after accounting for hyperinsulinemia, the association of T2D risk with LA, DGLA and AA was attenuated, suggesting that omega-6 fatty acids might be a marker of metabolic changes in pre-diabetes.

Omega-6 Fatty Acids as Biomarkers for Asthma

Asthma is a common respiratory disease characterized by chronic airway inflammation with the involvement of multiple cells (eosinophils, mast cells, T lymphocytes, neutrophils, etc.) and cellular components. Regulation of the inflammatory response is one of the important physiological functions of omega-6 fatty acids. Nested case-control studies in children showed that serum LA was associated with lower risk of asthma and better lung function (measured by FEV1), while AA levels were strongly associated with increased asthma prevalence and reduced FEV1 (Bolte et al. 2006). A case-control study analyzing erythrocyte membrane fatty acids in adults also reported that higher levels of membrane LA were associated with a lower risk of asthma (Broadfield et al. 2004). A Mendelian randomization study by Zhao and Schooling (2019b) also reported that genetically predicted higher LA was associated with a lower risk of asthma and lower levels of eosinophils and neutrophils (Table 2). In addition, corticosteroids are commonly used in clinical practice to treat asthma, and animal studies have shown that glucocorticoids could inhibit the release of AA from phospholipids and that any pharmacological effect of corticosteroids may lead to increased levels of LA in asthma patients (Blackwell et al. 1980). Therefore, changes in LA or AA levels may be of clinical value as a biomarker to guide medication in patients with asthma.

There are several possible explanations. LA may act through the derivatives PGE2 and LXA4 (Bonnans et al. 2002; Sastre and del Pozo 2012), which can inhibit

Table 2 Genetically predicted LA with asthma and white blood cell traits using Mendelian randomization

Exposure	Outcome	Source	OR/ Beta	95% CI	P value
Genetically predicted LA	Asthma	TAGC	0.70	0.58 to 0.83	6.8×10^{-5}
		UK Biobank	0.73	0.64 to 0.84	2.9×10^{-6}
		Both	0.72	0.65 to 0.80	1.2×10^{-9}
	Eosinophilic count	UK Biobank	−0.03	−0.061 to −0.004	0.02
	Neutrophil count	UK Biobank	−0.04	−0.057 to −0.023	3.6×10^{-6}
	Monocyte count	UK Biobank	−0.02	−0.046 to 0.008	0.16

From: The role of linoleic acid in asthma and inflammatory markers: a Mendelian randomization study

the proliferation of T helper cells and the production of various cytokines, such as IL-1, IL-2, and TNF-α, suppress the aggregation of pro-inflammatory cytokines and inflammatory cell, thus suppressing the airway inflammatory response (Namazi 2004). LA also has the potential to suppress asthma by increasing androgenic activity and promoting immune senescence (Gromadzka-Ostrowska 2006), as sex hormones have been shown to be beneficially associated with asthma (Scott et al. 2016). In addition, the conversion of LA to AA may be enhanced in asthma patients (Bolte et al. 2006). As a precursor of prostaglandin D2, AA contributes to and increases the production of pro-inflammatory cytokines and leukotrienes (Fajt et al. 2013), which leading to bronchoconstriction, increased vascular permeability and mucus secretion, and additional release of pro-inflammatory cytokines (Funk 2001). Patients with asthma have elevated circulating or tissue levels of AA and decreased levels of LA, reflecting ongoing airway inflammation, suggesting that omega-6 may serve as a potential predictive or diagnostic biomarker for asthma.

Omega-6 Fatty Acids as Biomarkers for Autoimmune Disease

Omega-6 fatty acids may also be used to predict common autoimmune disease, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple and sclerosis (MS). A metabolomics study comparing serum metabolites in 20 SLE patients and 9 healthy controls found that serum omega-6 fatty acid (LA, GLA, DGLA, and dihomolinoleate) were significantly lower in SLE patients than in controls (Wu et al. 2012). A case-control study of juvenile idiopathic arthritis (JIA) found no significant difference in dietary intake of omega-6 fatty acids between patients and controls, but serum AA levels were lower in patients, especially in patients with active and short-lasting disease, and serum total omega-6, LA, and AA levels were inversely correlated with the number of active joints, suggesting a protective association against JIA (Gorczyca et al. 2017). For rheumatoid arthritis (RA), de Pablo et al. (2018) conducted a nested case-control study in recent years and found that high erythrocyte levels of omega-6 fatty acid LA were associated with a reduced risk of RA in southern European. A Mendelian randomization study conducted by Zhao and Schooling (2019c) also found that genetically predicted LA was associated with lower risk of RA and SLE (Table 3). As such, omega-6 fatty

Table 3 Genetically predicted LA with RA and SLE using Mendelian randomization

Exposure	Outcome	Source	OR	95% CI	P value
Genetically predicted LA	RA	Rheumatoid Arthritis Consortium	0.96	0.94–0.98	<0.001
		Meta-analysis with UK Biobank	0.97	0.95–0.98	<0.001
	SLE	ImmunoBase Consortium	0.95	0.91–0.99	0.02
		Meta-analysis with UK Biobank	0.95	0.92–0.99	0.01

From: Role of linoleic acid in autoimmune disorders: a Mendelian randomization study

acids may predict SLE and RA. Their role in MA is less clear. RCTs did not show any beneficial effects of dietary supplementation with omega-6 fatty acids on relapse rates, disability progression, or disease activity in clinically active MS (Wergeland et al. 2012).

Omega-6 Fatty Acids as Biomarkers for Cancer

Cancer is the leading cause of global morbidity and mortality. The most common cancers include lung, breast, intestinal, and prostate cancers, accounting for 40% of diagnosed cancers in the world (https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer#heading-One (2019)). In vitro and in vivo animal studies have shown that AA might involve in cancer pathology (Sauer et al. 2007; Xu and Qian 2014), such as the proliferation of human breast cancer cell line BT-474 and lung cancer cell line A549 (Mouradian et al. 2014; Welsch 1992). In contrast, GLA, the derivative of LA, can inhibit the cell growth in vitro of human neuroblastoma cell lines and others (Fujiwara et al. 1986), selectively induce apoptosis in multiple human cancer cell lines including human breast cancer cells, lung cancer cells, and prostate cancer cells (Xu and Qian 2014), and dietary supplements of GLA can also reduce tumor growth in rat models (Colquhoun 2002).

Although studies in vivo or vitro have demonstrated the involvement of omega-6 fatty acids in pro-cancer or anti-cancer processes, the associations in epidemiological studies were inconsistent, showing inverse (Chavarro et al. 2007; Laaksonen et al. 2004; Rissanen et al. 2003) or positive (Harvei et al. 1997; Pot et al. 2008) associations. Kang and Liu (2013) suggested that the lack of conclusive evidence linking omega-6 to cancer risk may be because these studies only considered absolute levels of omega-6, rather than its ratio with omega-3 fatty acids. Omega-3 fatty acids compete with omega-6 for the same metabolic enzyme system, so they can limit the expression of omega-6-derived metabolites such as AA and leukotriene, and down-regulate the expression of growth factors (Kang and Liu 2013) (Fig. 3). The ratio of omega-3 to omega-6 is considered to be a predictor of cancer progression (Huerta-Yépez et al. 2016; Xu and Qian 2014). Results from European Community Multicenter Study showed that considering omega-3 or omega-6 levels in adipose tissue independently, there was almost no association with breast cancer risk, while the omega-3/omega-6 ratio displayed an inverse association with breast cancer risk (Simonsen et al. 1998). Comparing the fatty acid composition in adipose tissue in breast cancer cases and benign controls suggests that 18:3n-3/18:2n-6 and long chain omega-3/total omega-6 ratios exhibited a negative association with breast cancer (Maillard et al. 2002). Similarly, a case-control study showed a high omega-6/omega-3 diet ratio predicts the risk of prostate cancer, and possibly high-grade prostate cancer (Williams et al. 2011). A nested case-control study of Australian adults also reported that a high ratio of plasma omega-3/omage-6 concentrations was associated with a reduced risk of squamous cell carcinoma (SCC) (Wallingford et al. 2013). As such, it may be more informative to consider the ratios in the prediction of cancer risk.

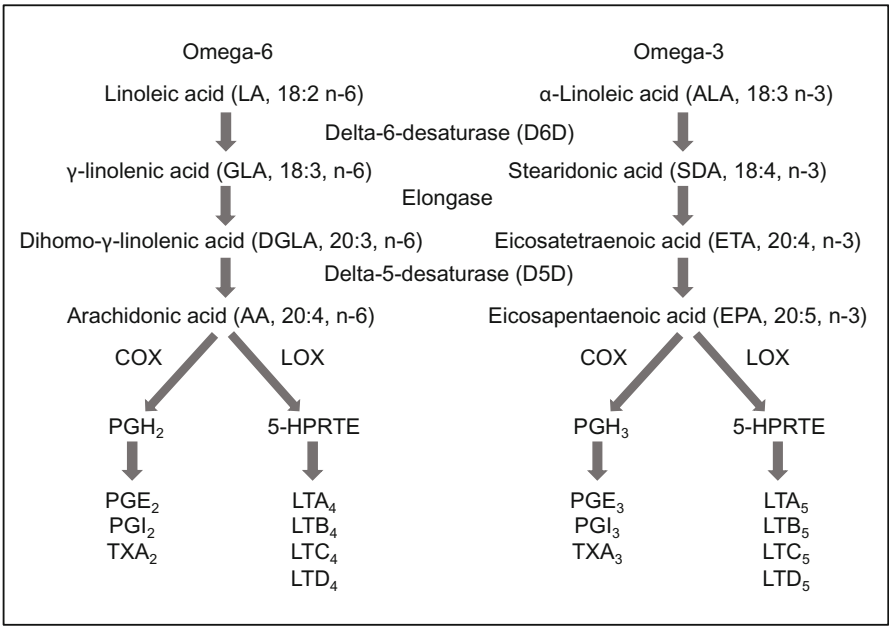

Fig. 3 Omega-6 and omega-3 fatty acid metabolism

Limitations on the Application of Omega-6 Biomarkers

Circulating or tissue fatty acids may predict dietary intake, while they are also influenced by other factors, such as smoking, alcohol drinking, BMI, physical activity and energy intake. Meanwhile, these biomarkers may not be sensitive to small amount of dietary fatty acid intake. In addition, the evaluation of omega-6 fatty acid biomarkers is usually expressed as a percentage of total fatty acids, which only reflects the relative intake of omega-6 fatty acids rather than absolute measure of total omega-6 fatty acid intake (Baylin and Campos 2006). As biomarkers for diseases, omega-6 fatty acids may predict cardiovascular disease, diabetes, asthma, autoimmune disease, and cancer, However, the associations with these diseases varied in different study designs, which needs more validation before clinical application.

Mini-Dictionary of Terms

- **Leukotrienes**. A class of eicosanoid inflammatory mediators derived from the metabolism of arachidonic acid in leukocytes, usually accompanied by the production of histamine and prostaglandins, and are important chemical mediators in Inflammation, allergies and immune diseases.

- **Mendelian Randomization study**. A method of data analysis that uses genetic variants as instrumental variables to examine the causal relationship between intermediate phenotype and disease outcomes. As the genetically determined risk factors are randomly allocated at born, MR can lower confounding bias by applying genetic variants as instruments.

Summary Points

- Omega-6 fatty acids are responsive to dietary intake and may be used in the assessment of dietary fatty acids intake in epidemiological and clinical studies.
- Omega-6 fatty acids are potential biomarkers for predicting cardiovascular disease risk.
- Omega-6 fatty acids are likely to be a marker of metabolic changes in pre-diabetes.
- Omega-6 may serve as a potential predictive biomarker for asthma.
- LA and its derivatives in omega-6 fatty acids have predictive value for autoimmune diseases such as SLE and RA.
- The ratio of omega-6 and omega-3 fatty acids may be considered as informative biomarker in the prediction of cancer risk.
- The use of omega-6 fatty acid biomarkers to assess dietary intake, and predict chronic diseases still has many limitations that prevent widespread apply in practice.

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Fatty Acid Profile of Red Blood Cells as Markers in Dietary Regimes and Beyond

20

Carla Ferreri, Anna Sansone, Alessandra Ferocino, Itziar Tueros, and Sara Arranz Martinez

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Abstract

The role of fatty acids as components of membrane phospholipids deriving from an appropriate combination between metabolism and nutrition is widely known and well assessed in biochemical, biophysical, biological, pharmacological, and medical research. The gap between this knowledge and applications as health biomarkers has become more and more evident as the fatty acid deficit appears among the most relevant causes of higher incidence of noncommunicable diseases. The review highlights some of the main aspects regarding fatty acids involved in the formation of membrane lipidome and explains the concept of fatty acid balance, which involves the fatty acid diversity, including dietary requirements, needed for the functioning of cell membranes in different tissues. Fatty acids furnish the best example of the interaction between nutrition, being components of dietary lipids and in particular essential fatty acids only provided by the diet, and metabolism through de novo synthetic pathways. For this reason, fatty acids are ideal biomarkers of the required balance between nutrition and metabolism. Membrane lipidomics is the modern approach to evaluate the appropriate dietary consumption and the metabolic performance, taking advantage of automatized and accredited analytical methodologies developed in view of creating big data and assessing the individual condition, applicable to personalized strategies in prevention and diseases.

Keywords

Membrane lipidome · Essential fatty acids · Omega-6 fatty acids · Omega-3 fatty acids · Fatty acid measurement · Lipidomics

Abbreviations

ARA	Arachidonic acid
ARDS	Acute respiratory distress syndrome
CE	Cholesteryl ester
DGLA	Dihomo-gamma linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
ESI/MS-MS	Electrospray mass-mass spectrometry
FA	Fatty acids
FAD1	Fatty acid desaturase 1
FAD2	Fatty acid desaturase 2
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
HDL	High-density lipoproteins

LCAT	Lecithin cholesterol acyl transferase
LCP	Lysophosphatidylcholine
LDL	Low-density lipoproteins
MHO	Metabolically healthy obesity
MONW	Metabolically obese normal weight
MUFA	Monounsaturated fatty acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
SFA	Saturated fatty acid
SM	Sphingomyelin
TG	Triacylglycerol
VLDL	Very-low-density lipoproteins
WB	Whole blood

Introduction

Lipids exist in a variety of foods and serve as an energy source with high caloric density, adding texture and taste and contributing to satiety. Besides the dietary issue, being the third class of macronutrients, lipids play fundamental roles in the formation of biological structures and in many metabolic processes of living organisms. They support cell structures and functioning and assist in essential processes such as energy reserve, regulation, and production of certain hormones including estrogen, testosterone, and cortisol, transmission of nerve impulses, signaling, and transport of fat-soluble nutrients (Spector and Yorek 1985).

Examining lipids, they are structurally divided into two main classes: fatty acid-containing lipids and sterol-containing lipids. We will concentrate on the first ones, which are quantitatively important and also because fatty acids furnish the best example of the interaction between nutrition and metabolism, being components of dietary lipids especially those provided exclusively by the diet (essential fatty acids, EFA) and being also formed by de novo synthesis, such as saturated and monounsaturated fatty acids (SFA and MUFA). Fatty acids can express the quantitative and qualitative balance between nutrition and metabolism. In particular, fatty acids acquire a crucial biological meaning as the hydrophobic components of phospholipids, necessary building blocks for cellular functions and for the existence of cells and subcellular organelles, with the primary role in the formation of the membrane lipid bilayer (Casares et al. 2019). The chemical structures of fatty acids (FA) that form phospholipids express a diversity with the characteristic membrane FA profiles for each tissue, made of saturated, monounsaturated, and polyunsaturated fatty acid chains (SFA, MUFA, and PUFA), related to the specific cell functions (Glatz and Luiken 2015; de Carvalho and Caramujo 2018). In Fig. 1 the three families of fatty acids are shown, with the most relevant SFA, MUFA, and PUFA members. Studies

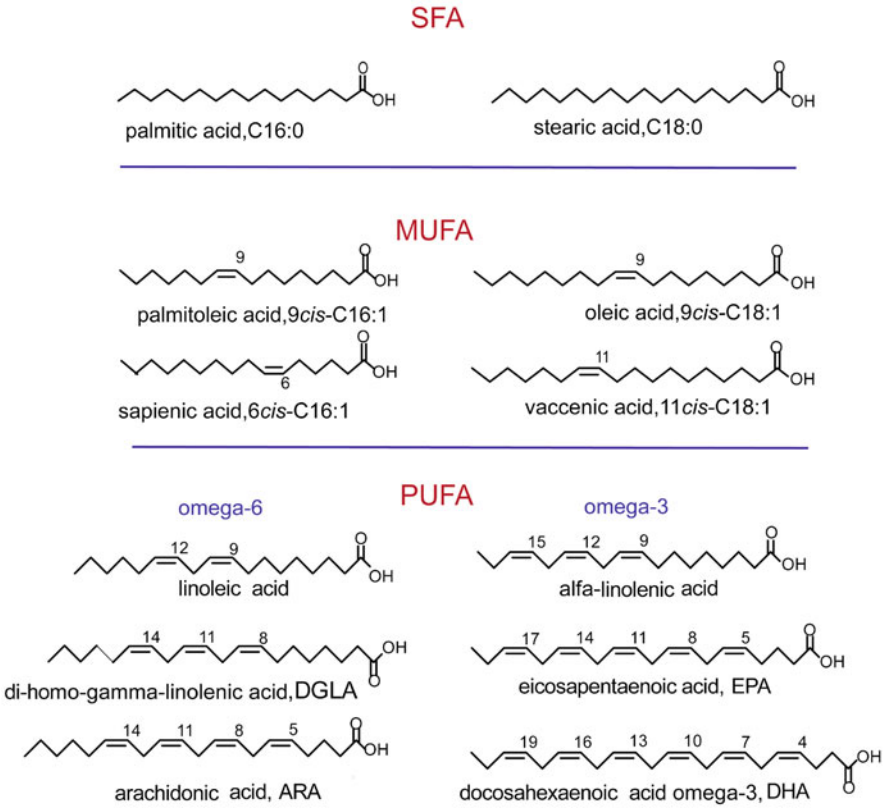

Fig. 1 The three fatty acid families and examples of the main fatty acid structures

of membrane biophysics demonstrated that the saturated/unsaturated FA ratio regulates fluidity and permeability properties with predominant effects on the whole cell functioning and fate (Ibarguren et al. 2014; Harayama and Shimizu 2020; Levental et al. 2000). The presence of SFA renders the membranes thicker and less fluid due to the strong packaging of the saturated chains; also, the packing defect can be correlated to the degree of membrane hydrophobic area exposed to aqueous environment. PUFA moieties decrease membrane rigidity, increasing fluidity and permeability; however, they also increase chemical reactivity for their sensitivity to oxidative and isomerization processes (Chatgilialoglu et al. 2014; Christie and Harwood 2020; Vetica et al. 2020). The excessive oxidation of lipid is a very well-known process involved in aging of living organisms (Johnson and Stolzing 2019) and recently associated to a specific cell death defined ferroptosis (Yang et al. 2016; Yan et al. 2021), whereas the conversion of the natural cis geometry to the unnatural trans geometry of fatty acids has been associated with several membrane dysfunctions and increased health risks (Islam et al. 2019).

Overall, the FA balance is considered largely responsible for the cell capacity to respond to external stimuli: membranes work as sensors, orchestrating receptors for external signals and even participating themselves to the signal transmission to nuclei, in order to react immediately with proper responses and adaptation. Balance in the cell membrane FA composition leads to the balance of the functions in each cell and thus in the organism as a whole (Calder 2015). In the seminal review of Harayama et al. (Harayama and Riezman 2018), membrane lipid composition diversity is clearly described, highlighting the phospholipid fatty acids for different tissues, and correspondently, enzymes are regulated by tissue-specific transcriptional factors with a variable distribution. An interesting example is reported evidencing the most present fatty acids occurring in the sn-2 position of phosphatidylcholines in the corresponding tissues: oleic acid is present in the brain, palmitic acid is present in the lung, linoleic acid (EFA omega-6) is present in the liver, and docosahexaenoic acid (DHA) (omega-3) is present in the heart, while arachidonic acid (omega-6) is present in different concentrations in all analyzed tissues. This means that cell membranes are under homeostatic control and alteration of these compositions, due to the unavailability or insufficient intake of such molecules, can be correlated to different diseases.

This review will focus on crucial information on fatty acids to evaluate human health conditions related to nutrition and metabolism, highlighting the role of cell membrane as comprehensive biomarker to check the natural biological balance.

Lipids in Nutrition

Evaluating Fatty Acids in Tissues and the Impact of PUFA

To evaluate the impact of dietary fats at the level of various tissues, the most thorough study was provided by Abbott et al. (2012), examining rats treated with 12 different diets of variable SFA, MUFA, and PUFA contents for a period of 8 weeks. The use of a murine model allowed for the examination of the following tissues: muscle, heart, brain, liver, and RBC membranes, as well as of plasma lipid contents; in all tissues, phospholipids were analyzed, while triglycerides were monitored only in plasma and adipose tissue. The results evidenced that in tissues membranes were not strongly affected by moderate fat contents in diets, apart from plasma and adipose tissue; consequently, tissues can be used to monitor both dietary and metabolic influences. Tissues can have problems in achieving the normal fat composition because of the lack of intake of appropriate fatty acids, and this deficit can induce a higher response to dietary fat intakes in order to restore the unbalance. In particular, red blood cell (RBC) membrane was considered the best homeostatically regulated tissue, where SFA and MUFA contents do not vary for short-term dietary changes, and PUFA content in RBC membranes can reliably reveal the PUFA intakes. It was also suggested to use the PUFA balance index (omega-3/omega-6 + omega-3 PUFA) as better indicator with respect to the omega-

6/omega-3 ratio, in order to evaluate the correct PUFA intakes. In particular, the authors evidenced the PUFA balance threshold as <10% in their murine model, which means that below such membrane PUFA levels a direct influence and amelioration can occur by appropriate dietary conditions, whereas poor influence from the diet was noted when PUFA balance is >10%. We evaluated such PUFA balance for humans to be similar (<11%) (Ferreri et al. 2016), and we believe that such an index is useful to determine the success of dietary interventions, as well as it can be in future used to establish the impact of lipid nutraceutical supplementations.

It is worth noting that from the murine model and the examination of all tissues (muscle, heart, brain, liver, and RBC membranes), RBC membranes were highlighted as the best tissue for evaluating the metabolic/dietary effects. This is an important information to transfer to diagnostics for humans.

As mentioned in the Introduction, lipids and fatty-acid-containing lipids are fundamental elements of a balanced diet. The effects of different fatty acids taken from nutrition on human health are reported in population studies and were covered by several reviews and books (Clifton and Keogh 2017; Nettleton et al. 2017; Afshin et al. 2019). In the last years, the use of molecular tools (metabolomic, nutrigenetic, metagenomic, etc.) has provided new scientific evidence related to the connections of phenotypes, diet, and metabolism (Sun and Hu 2016; Demetrowitsch et al. 2020). This knowledge can be useful for personalized therapies and contributes to providing more precise nutritional recommendations, mainly for an adequate fat quality and intake for different age groups and health conditions (Corella and Ordovás 2018; Mills et al. 2019; Picó et al. 2019). In this sense, personalized intervention strategies could provide precise nutritional guidance and contribute to successful long-term interventions (Teixeira et al. 2015). Even though dietary guidelines for macronutrients differentiated by age or gender are established (EFSA Panel on Dietetic Products and Allergies 2010; Fao 2010), according to the scientific evidence on requirements of different population groups, especially from energy intake, interventions to control metabolic diseases are not specific or differentiated regarding the intake of food groups or specific nutrients. The use of molecular tools can provide new scientific proofs related to the characterization of different phenotypes together with the impact of diet on metabolism (Camilleri and Staiano 2019).

The Importance of Omega-6 and Omega-3 Fatty Acids as Biomarker of Intakes from Nutrition

Seminal review works reported the importance of omega-6 and omega-3 PUFA in nutrition (Simopoulos 2016; Brown et al. 2019; Hanson et al. 2020; Djuricic and Calder 2021). These are EFA, as mentioned in the Introduction, and their precursors must be taken from the diet. In Fig. 2 the two pathways of omega-6 and omega-3 FA formation are shown, departing from linoleic acid and alpha-linolenic acid, for omega-6 and omega-3, respectively.

Although the dependence of humans from dietary lipids for PUFA synthesis is well assessed, this knowledge did not yet translate to introducing them as biomarkers

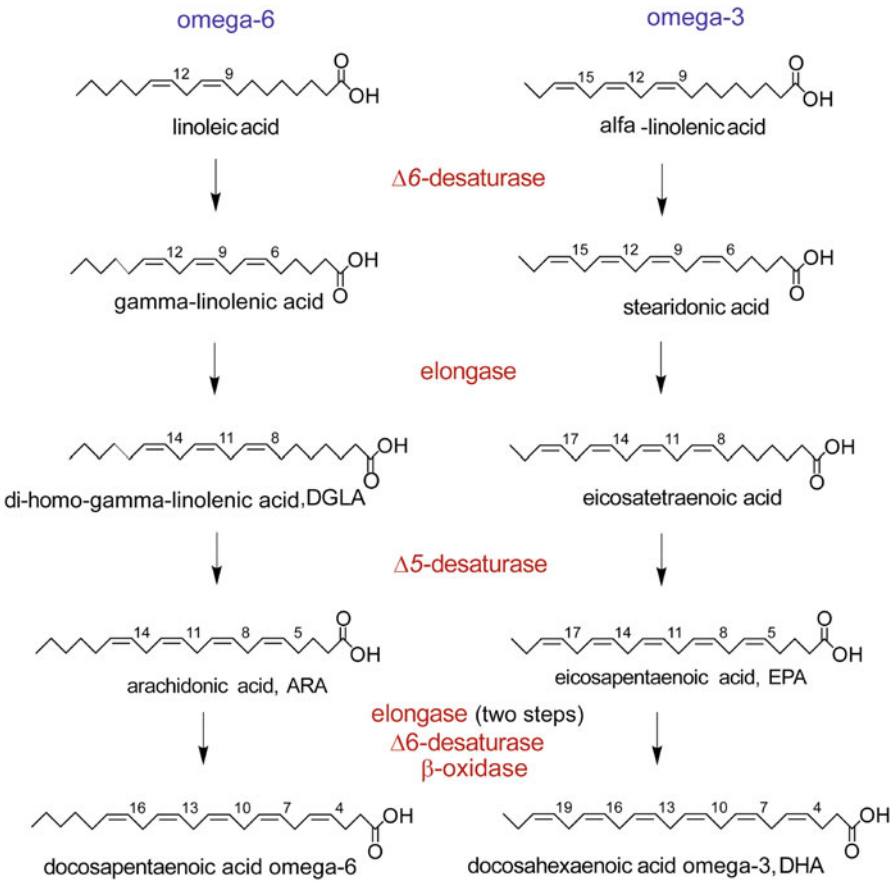

Fig. 2 The two pathways of omega-6 and omega-3 fatty acids starting from their dietary precursors

of balanced intake for clinical use. The introduction of PUFA measurement is reasonable, in order to check the levels in the body and individuate deficit, which can be harmful and create pathological conditions. Moreover, as explained in the previous section, fatty acids in membranes are the best sample to analyze, due to their complete information on nutrition and metabolism. It is worth mentioning that efforts have been made toward the best analytical tools to perform such analysis (Ferreri et al. 2016).

What are the main evidences between fatty acids as healthy elements reaching specific levels in the composition of membranes? Two reports have been provided recently:

– In the Global Burden of Disease Study (Afshin et al. 2019), among preventable risk factors for noncommunicable diseases (NCDs), there are fatty acid intakes, i.e., a health risk is produced when the diet is low in seafood omega-3 fatty acids

(considering the mean daily intake of EPA and DHA is less than 250 mg/day) and in PUFA omega-6 fatty acids from all sources (mainly liquid vegetable oils, including soybean oil, corn oil, and safflower oil, which is less than 11% of total daily energy) (Afshin 2019). As an example, in the panel of the different countries, the intakes of omega-3 in Western Europe resulted to be below the threshold for omega-3, thus showing that it is not malnutrition that is the only cause of low intakes of essential elements.

– The second global evaluation was performed on omega-3 in RBC as biomarker of the intakes, using the omega-3 index, i.e., the percentages of EPA and DHA in the RBC membrane phospholipids (Stark et al. 2016). Such index has a threshold for high risk of cardiovascular disease incidence <4% and was found to be <6% in several countries and in different continents, which corresponds to medium-high risk. In our studies on RBC membrane phospholipids in humans, we found decreased levels of omega-6 and omega-3 FA in RBC membranes in several health conditions, including parenteral nutrition, liver diseases, menopause, obesity, cancer, and autism (Sansone et al. 2013; Giacometti et al. 2017; Pironi et al. 2017; Svegliati-Baroni et al. 2019; D'Alberti et al. 2020; Jauregibeitia et al. 2020) both in Italian and Spanish populations. From our results and others, it emerges that it is time to bring this molecular information of the RBC lipidome into clinical protocols and medical practice.

Omics Technologies and Membrane Lipidomics

The Information Provided by "Omics" Technologies

Precision nutrition aims to develop more complete and dynamic nutritional recommendations based on changing parameters, interacting with an individual's internal and external environment. The scientific community shares that the future of precision nutrition should not just be based on nutrigenetics but also include epigenetic factors, such as eating habits, eating behavior, physical activity and environment, microbiota, and metabolism (Ferguson et al. 2016; de Toro-Martín et al. 2017), individuating new integrated biomarkers. Then, the measurement of the interaction between diet and these biomarkers is of great interest to give precise nutritional recommendations. The "omics" technologies find ideal application in this context. In the past two decades, the ability to study cellular and molecular systems has been transformed through the development of omic sciences. Omic sciences aim at the collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms (Vailati-Riboni et al. 2017). In nutrition, metabolomics can be applied to characterize metabolite profiles, which define the molecular transformations of bioactive compounds, discover status biomarkers, and allow the monitoring of dietary interventions studies (Corella and Ordovás 2015; Odriozola and Corrales 2015; Sébédio 2017). Research on this subject highlighted that a better understanding of interindividual variability in response to diet is needed, in order to design nutritional

strategies adapted to the age, type of pathology, and food preferences of each individual. In the era of nutrition and precision medicine, the search for new noninvasive biomarkers and gold standard techniques is directed toward integrative markers that reflect not only nutritional status but also connect with metabolism, and therefore reflect the connection between nutrition and health status (Jacob et al. 2019; Misra 2020). The central problem is that appropriate biomarkers are not yet available for many health disorders, and those available require highly invasive or complex methodologies that have, therefore, high impact from an economic point of view.

Lipidomics and Membrane Lipidomics

Lipidomics was first described by Han and Gross (Han and Gross 2003) as a branch of metabolomics that consists of a qualitative and quantitative analysis of the lipidome, which refers to whole lipids in cells. Apart from identifying and quantifying all lipids, lipidomics focuses on all the metabolic interactions moved and related with lipids, along with protein expression and gene regulation in response to a stimulation or disturbance. At the same time, analysis of a lipid profile allows for the evaluation of a disease onset and its progression, enabling, by measuring the quantification of the lipidome alterations, the personalization and monitorization of the treatment (Gong et al. 2020). Integration of the lipid profile through the use of multivariate statistics can be helpful to discover potential biomarkers, by understanding disease pathology, and the mechanisms of lipid-mediated disease (Hu et al. 2009). As metabolites are considered direct products of biochemical processes, they are easily correlated with the studied phenotype. Lipidomics took advantage from the advances in analytical technology, including mass spectrometry (MS)-based shotgun lipidomics and MS coupled with separation technique-based lipidomics that can be applied to characterize and discover the role of lipids in cellular functions and disease biomarkers in several pathologies (Zhao et al. 2015). These advances are not trivial, since they have opened the doors to a robust analysis of different lipids, facilitating a better understanding of lipid metabolism (Hu et al. 2009). A major advantage of such lipidomics-based discovery is that, together with our relatively good understanding of many biosynthetic and metabolic pathways, it will lead to the identification of pathways and enzymes (and enzyme modulators).

Membrane lipidomics was also developed by several groups, including our own, focusing on the molecular composition of this important cellular compartment where the fatty acid quality and quantity regulate fundamental functions, as mentioned in the sections above. Membrane lipidomics is not only related to metabolism but also to dietary intakes. Moreover, it does not represent only metabolic and biosynthetic pathways but also expresses the effects of the biophysical forces, which spontaneously create the phospholipid assembly and generate an equilibrium of structures and functions as that of cell membranes. From this point of view, the molecular information obtained from the analysis of membrane lipids is very rich and still waiting to be fully evaluated. As a matter of fact, membrane lipidomics cannot be considered in the

same frame as metabolomics, since its fatty acid content contains information that is applicable as a comprehensive biomarker panel, to evaluate cell molecular status and equilibrium, reflected in the health status with its metabolic and dietary contributions.

As explained above, the unbalance in the composition and structure of cell membranes has been associated with pathophysiological aspects in humans (Escribá and Nicolson 2014), also considering that the adequate lipid balance in cells is necessary to control the immune system and inflammation (Escribá 2017). Correlations between lipids and their variations that occur in pathological states with the respective exogenous influences that act on them and the endogenous consequences on the metabolism are important to deepen the molecular mechanisms underlying the regulatory roles of lipids for the membrane lipid structure and membrane-related signaling events. Although the fatty acid composition of each tissue is specific, it can be inferred that mature RBC is a good reporter of the metabolic state of different organs, tissues, and cells: mature RBC is a long-term circulating cell (120 days of mean lifetime) and its membrane reflects an overall status of the diet-metabolism interaction of each individual. Information from such RBC profile can be used to design personalized and precise nutritional and nutraceutical strategies, mainly for fatty acid intake. Sansone et al. (2016) (Sansone et al. 2016) highlighted in their work the importance of C16 MUFA isomers in RBC as emerging metabolic marker in morbidly obese patients. Then, Jauregibeitia et al. (2020) and Amezaga et al. (2018) published the RBC fatty acid profile characteristic of children with obesity and cancer patients, respectively, and compared them to people without disease. In a recent study (Amézaga et al. 2021), certain omega-6 fatty acids in the red blood cell membranes were correlated with chemosensory alterations in breast cancer patients. Linoleic acid showed negative correlation with xerostomia (dry mouth), while arachidonic acid correlated positively with salty taste alteration. The involvement of these polyunsaturated lipids suggests the importance of oxidative and nutritional conditions of cancer patients, pointing out the lipid profile in the red blood cells as potential biomarkers for nutrition in cancer patients.

The combination of individual or multiple fatty acids can indeed provide important molecular biomarker of pathological conditions to be followed up during pharmacological and nutritional therapies.

It must be evidenced that one of the greatest difficulties lies in the ability to design nutrition-based strategies that reach the cellular and molecular levels to control or prevent diseases. In this sense, membrane lipidomics can be a useful tool to integrate in “omics” panels to be applied in medicine and precision nutrition, characterize specific profiles, and follow up molecular changes of the membrane status, proving that the intervention is able to address the needs of individuals and population groups in a personalized way (Boja et al. 2014). Indeed, one of the practical applications of lipidomics, in this sense, has been identified as membrane lipid therapy, a new perspective based on the development of molecules and formulations that regulate membrane lipids in order to treat different diseases such as cancer, neurological pathologies, metabolic disorders or cardiovascular diseases, and many others (Ferreri et al. 2016; Escribá 2017). All these factors make the measurement of the lipids that constitute the cell membrane a major point of interest.

The Development of RBC Fatty Acids as Biomarkers

The Importance of Sampling Procedure, Analytical Recognition, and Evaluation of Biological Significance

An important aspect is the determination of the best sampling protocol for membrane fatty acid characterization. It is evident that the choice of noninvasive testing should be the first issue to be addressed. Nevertheless, the importance of choosing a sample where the cellular presence and functions are significant for molecular information is also evident. The difficulty of finding a reliable fatty acid biomarker can be exemplified by the work of Chowdhury et al. (2014) regarding meta-analysis of studies on the association of dietary, circulating, and supplemented fatty acids with coronary risk. The findings did not support cardiovascular guidelines for the exclusion/use of specific quality of fatty acids from dietary consumption, since there is not statistically significant association between high consumption and a supplementation of ω-3 and ω-6 polyunsaturated fatty acids and a lower risk for coronary outcome. Furthermore, there is no evidence, using dietary intake and circulating biomarkers, that a reduction of total saturated fatty acids can be connected with a reduction of cardiovascular disease. It should be said that analysis and interpretation of results have been complicated by several limitations: the moderate amount of available data on some specific circulating fatty acids and subsequent possible over-/underestimations of associations, the lack of serial assessment of fatty acids in the same persons, and the impossibility to consistently adjust for potential confounding factors across all studies (ex. differences in study design, population characteristics and dietary habits, sample type, composition of supplementation regimes, trial duration and power). We can also evidence other critical points of population studies like those regarding analytical methodologies or the type of fatty acid supplementation: the choice of analytical method is crucial, since separation and recognition of the different fatty acid structures must be achieved. A relevant example is given by our experience on the libraries of MUFA and PUFA trans fatty acids and their identification in human specimens. We contributed to this aspect by affirming gas chromatography (GC) under specific conditions as the gold standard for fatty acid analysis, combined with the chemical derivatization of the unsaturated fatty acids as dimethyl disulfide derivatives, in their turn separated by GC and recognized by mass spectra (GC-MS) to unequivocally establish the position of the double bond in their structures (Sansone et al. 2013, 2016; Ferreri et al. 2020a). By such accurate analytical protocol, interesting investigations were performed with the discovery of biomarkers of a new biochemical pathway regarding palmitic acid involving the formation of the n-10 MUFA known as sapienic acid, with the double bonds in position 6–7. This is a positional isomer of palmitoleic acid that has the double bond in 9–10 (see Fig. 1); as mentioned above, our group focused on the C16 fatty acid isomers recognized by an accurate lipidomic protocol combining synthetic and analytical approaches (Sansone et al. 2016; Scanferlato et al. 2019; Ferreri et al. 2020a).

Other two critical points can be evidenced in the studies and meta-analyses run so far: a) the first issue regards the presence of fatty acids in different biological compartments and cells, presenting different biological and metabolic meanings. Therefore, it is not correct to combine studies done in plasma, which contains circulating lipids, with those performed in adipose tissues or cellular fractions taken from blood; b) the supplementation of fatty acids as oils, capsules, or emulsions as well as their dosages (from hundreds of milligrams to several grams) deeply influences the distribution and the occurrence of oxidative and chemical processes on these molecules, especially PUFA. The relevance of these points influences the rightness of the therapeutical approach according to patients' conditions. In particular, the identification of the specific needs of the patients must be carried out before treating them with a fatty acid supplementation, and, after administration, assessment of bioavailability and metabolic fate must be included in the study. Pitfalls on these aspects that can be observed in the published studies can have possibly influenced, under-evaluated, and, even, confused the identification and the intrinsic value of fatty acids as biomarkers.

The Choice of Red Blood Cell Membrane and Comparison with Other Blood Compartments

Studying the literature, we identified some specific points and worked to obtain a significant result in the identification of the red blood cell membrane as repeatable and reliable biomarker panel of fatty acids. RBCs are the most numerous cells formed every day (>70% of our cells formed every day are RBCs) (Sender et al. 2016) so that the lipid pools present in the body are abundantly used for forming RBC phospholipids. The RBC membranes need an appropriate mix of fatty acids to be performant in exchanging oxygen and nutrients with all tissues; therefore, the presence of unsaturated moieties and the levels of MUFA and PUFA are very important molecular requirements for their functioning. In this section other relevant issues regarding RBC will be underlined. The mean lifetime of RBC is 120 days and in the "mature" phase the cell diameter and the density change, so they can be useful parameters to obtain a cell selection without adding other materials.

It is worth to highlight that a strong effort was also put in the laboratory and analytical methodologies that are necessarily to be automatized in order to reduce manual operations and errors. Moreover, another important tissue, also referring to the need of big data for health care, is that the laboratory procedures and analyses are certified by the national bodies of accreditation, in order to have a unified platform that every laboratory can perform and receive auditing for their performance. We were strongly involved in the automatization and accreditation of the methodology from the blood separation of mature RBC to the final GC analysis, and realized a project that combines science and innovation at our research centers. Here below there are some important consideration from the literature that basically oriented the choices of our methodology and the focus on the importance of RBC membranes.

Several studies based on the comparison of fatty acids coming from plasma lipids, whole blood (WB), and RBC are performed, as the research of Risé et al. (2007). The results showed a different distribution in different compartments. The FA composition of WB contains the contribution of plasma lipids and RBC; therefore, the FA profile results from the FA present in lipid pools of plasma (circulating) and cells (structural).

FA profiles in plasma, LDL, and HDL show that they are similar but differ from those RBCs and platelets; the amount of essential fatty acid linoleic acid, coming exclusively from the diet, is higher in plasma and lipoproteins than in blood cells because they contain apolar lipids like triglycerides (TG) and cholesteryl esters (CE); in particular the high content of linoleic acid or arachidonic acid in CE is correlated with the selective activity of the enzyme lecithin-cholesterol acyltransferase (LCAT) that transfers FA from position 2 of PC to cholesterol giving the corresponding cholesteryl ester (CE) (Jonas 2000).

In cell membranes, PUFAs, both of the omega-6 and omega-3 families, express the dietary intakes and also the ability of metabolic transformations from triglycerides to phospholipids, thus referring to a long-term and stable accumulation for the membrane formation. The class of phospholipids, either in plasma and RBCs, is quantitatively the major class in determining the fatty acid blood profile and also the physiopathological status, being the class less affected by short-term dietary intake of the days or hours immediately before the analysis. Fatty acid content in phosphatidylcholine (PC), the principal phospholipid in RBC, shows that SFA, MUFA, and PUFA levels differ from the distribution in plasma: specifically, SFAs are the major component, while PUFAs are more similar to plasma HDL contents, indicating that there is indeed a selective exchange between membranes and this compartment (Marks et al. 1960; Reed 1968).

Hu et al. (2017) reported the conversion ratio of omega-3 between plasma and erythrocytes using a meta-analysis of 56 published studies. They applied meta-regression methodologies in order to take into account also age, sex, and study design. The conversion ratios from plasma PL to RBC of EPA, DHA, DPA, and total omega-3 were equal to 0.75, 1.16, 2.32, and 1.22, respectively; these values indicate a different transfer of fatty acids between plasma PL and RBC membrane PL, and could be useful to evaluate the effects of nutritional strategies when only one of these lipid type has been used. Conversion ratio can be used for nutritional strategies to determine the fatty acid profiles of different population coming from different epidemiological studies to allow for data collections and lipid pools from different methodologies.

The reflection of long-term fatty acid intake in RBC compared with plasma was the object of several studies of healthy and pathological subjects (Sun et al. 2007; Harris et al. 2013; Koehrer et al. 2014). The nutritional contribution in RBC was particularly monitored also in children and adolescents in order to follow the long-term implications and metabolic consequences in membrane RBC (Cortés et al. 2013); in the study, values of fatty acids were determined in healthy children in serum and in RBC membranes; in particular, the PUFA omega-6 linoleic acid was higher in serum with respect to arachidonic acid in membrane phospholipids; the

PUFA omega-3 EPA was higher in serum, while DHA was higher in membrane phospholipids. Interesting researches are based on the influence of lipoprotein alteration on RBC fatty acid profile: Deon et al. (2017) provided data on the RBC phospholipid FA composition and serum biomarkers in Italian children and adolescents with primary hyperlipidemia; the authors highlighted an inverse correlation between MUFA in RBCs and serum cholesterol or HDL-cholesterol/triglyceride ratio; the omega-6 PUFA in RBC was positively associated to serum HDL-cholesterol levels and inversely to dietary cholesterol; the total content of omega-3 was <4% in RBC.

Also, the contribution of genetic and environmental factors was studied to determine the correlation between FA profile and lipoproteins. Some studies evidenced the role of genetic factors in phenotypic covariation of omega-6 PUFAs and MUFAs with TG and VLDL (Jelenkovic et al. 2014). In this scenario, the polymorphism of FA desaturases comes into play, modulating blood and tissue lipid profiles. Specifically, FADS1 and FADS2 genes have been associated with PUFA levels in both serum phospholipids and RBC membranes (Schaeffer et al. 2006; Malerba et al. 2008).

On the other hand, some studies did not stress differences expressed by different blood compartments, unifying the meaning of FA composition of plasma, platelets, and erythrocyte lipids. This led to propose the FA analysis of whole blood as an approach for the FA status assessment. In the seminal review by Brenna et al. (2018), it has been thoroughly discussed that differences among the blood compartments are instead evident. On this basis and also considering the metabolic and structural roles of fatty acids in lipid classes, as discussed in this review, we believe that an important point in the assessment of fatty acids as biomarker of nutrition and beyond is the choice of the sample to examine for the outcome of the lipidome analysis: the dried blood spot analysis, composed of plasma and blood cells, is interesting due to its easy applicability in epidemiological studies and clinical trials, but the fatty acid profile analysis cannot be done since it does not correspond to a precise compartment, being a mix of circulating and structural lipids. To create a profile, the cell membrane compartment provides a well-defined cluster of lipids with structural roles, and this ensures the precision of the molecular information of fatty acids isolated from RBC.

The Proof of the RBC/Lipoprotein Fatty Acid Exchange

The potential exchange between RBC and plasma is an interesting topic, studied in an old work (Reed 1968) using ^{32}P, in vitro and in vivo, in humans and in dogs. In particular this research described the dynamism of phosphate moiety of four individual major phospholipid classes of circulating erythrocytes: sphingomyelin (SM), lecithin, phosphatidylethanolamine (PE), and phosphatidylserine (PS); phospholipid exchange between plasma and erythrocytes, coming from the in vitro experiments, was comparable with the observations in vivo. Lecithin and SM of RBC are exchanged with the corresponding plasma classes in the following percentages:

60% and 30% respectively, while phosphatidylethanolamine and phosphatidylserine appeared stable in red blood cell membranes. Also, in another old work by Renooij et al. (Renooij and Van Golde 1976), the temperature effect on phosphatidylcholine (PC) translocation process across RBC membranes and plasma-RBC phospholipid exchange applying ^{32}P isotope methodology in rats were investigated.

More recently, Dushianthan et al. (2019) studied in vivo turnover and dynamic flux of phospholipids between plasma and erythrocytes in healthy humans and subjects affected by respiratory disease (ARDS). This alteration was characterized by a high level of broncho-alveolar lavage fluid rich in pro-inflammatory lipid mediators derived from arachidonic acid. The authors used isotopes of choline and electrospray mass spectrometry (ESI-MS/MS) as analytical method to investigate individual molecular composition and dynamic exchange of PC, SM, and lysophosphatidylcholines (LPC) between plasma and RBC. In ARDS patients, significant alterations were evidenced in PC molecular composition, coupled with a continuous loss of arachidonoyl-PC.

Infusion of isotopic *methyl*-D9-choline resulted in the enrichment of labeled choline into plasma PC with consequent incorporation of erythrocyte PC, although it was much slower than plasma. The patients showed faster and higher enrichment of all phospholipids and lysophospholipids evidencing an increased flux between plasma and erythrocytes.

Baur et al. (2000) monitored the differential incorporation of fatty acids in muscle, erythrocyte, and adipose tissue membrane phospholipids and adipose tissue triglycerides in young children; further the effect of diet in infants was followed. The authors evidenced a significant positive correlation between muscle and RBC in docosahexaenoic acid (DHA) and total omega-3 content and the omega-6/omega-3 ratio. Adipose tissue triglycerides showed lower levels of long-chain PUFA omega-6 and omega-3 and high levels of MUFA than muscle and erythrocyte phospholipids. The fatty acid profile of RBC was a reasonable index of muscle DHA, total omega-3 PUFA, and omega-6/omega-3 ratio; breastfeeding showed a potent effect on fatty acid composition of all these tissues and high levels of PUFA omega-3 were detected especially in muscle and erythrocytes. This work evidenced an important aspect on sampling methods and their invasiveness: the withdrawal of blood is much less invasive and with few ethical problems with respect to biopsy procedures necessarily applied for muscle and adipose tissue sampling.

Regarding this issue, Di Marino et al. (2000) suggested to bypass the difficulties related to muscle biopsies by getting information on fatty acid composition using the easier accessibility of RBC. In order to sustain their thesis, the authors evaluated the phospholipid fatty acid composition of erythrocytes and muscles coming from abdominal surgery. In comparison with erythrocyte membranes, muscle membranes showed a significantly higher proportion in omega-6 PUFA specifically linoleic acid and lower SFA and MUFA. The results being significantly different in two compartments, the authors concluded that fatty acid composition coming from muscles cannot be extrapolated from the fatty acid profile of red blood cells. The authors retained this unexpected result since the fatty acids utilized by two analyzed tissues are derived from the diet of the same subjects via plasma; however, as indicated in

the first section of this review, it must be taken into account that specific differences are characteristic for each tissue; therefore, the choice of representative tissue for all the other non-easily withdrawn ones must be correctly extrapolated. One of the extrapolations concerns PUFA levels that are present in the RBC membranes and indeed represent the availability of the fatty acid pool for all the other tissues in the body. The RBC deficit of PUFA can be interpreted as a "systemic" deficit which impacts on all tissues in the subject, and on this basis the previously mentioned omega-3 index and PUFA balance can be properly developed as biomarkers for nutrition in clinical applications.

Ferreri et al. (2016) highlighted the powerful tool of membrane lipidomics using red blood cells and related information, due to the involvement of fatty acids in biophysical and biochemical processes, in signaling transduction and modulation of epigenetic pathways of cells. Red blood cell phospholipid membranes represent an interesting compartment to investigate because fatty acids represent a cluster and can be monitored for their changes in relation with lifestyle, nutrition, metabolism, and stress. As already discussed, the strong differences detected in plasma lipidomics are influenced by the diet and other factors that happened before the withdrawal or in a short-time relationship, and fatty acids of red blood cell membranes represent a stable information connected to metabolism and long-term dietary habits. The lipidomic application to mature RBC membrane is the closest approach to get information on the quality and quantity of fatty acids derived from metabolic and nutritional contributions, including remodeling and exchange processes. The content of fatty acids can be expressed as the balance of SFA, MUFA, and PUFA (omega-6, omega-3); a cohort of 10 representative fatty acids belongs to these families: palmitic and stearic acids for SFA; palmitoleic, oleic, and cis-vaccenic acids for MUFA; and linoleic, dihomo-gamma linolenic acid (DGLA), and arachidonic acid (ARA) for PUFA omega-6 and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for PUFA omega-3. In this review the PUFA balance index (omega-3/PUFA) has been mentioned, calculated on a cohort of healthy and diseased subjects, evidencing that a value of PUFA balance of 11% is a lower limit in humans that can be ameliorated with dietary treatment and nutraceutical supplementation to reach the proper fatty acid membrane balance.

Applications to Prognosis, Other Diseases, or Conditions

Application of C-16 Monounsaturated Fatty Acids in Cancer Diagnostics

In this section we wish to highlight the emerging role of the MUFA sapienic acid (6 cis 16:1) as a novel biomarker in cancer. It was recently indicated as a marker of cancer plasticity being present in high content in several cancer cell lines, mouse hepatocellular carcinoma, and primary human liver and lung carcinomas (Vriens et al. 2019). Sapienic acid is derived from the desaturation of palmitic acid by the action of FADS2 or desaturase delta-6; it is a positional isomer of 9 cis-16:1 (palmitoleic acid) and 7cis-16:1 and the presence of C16 isomers was first reported

by our group (Sansone et al. 2016); the unambiguous analytical recognition of C16 isomers is crucial for the determination of biochemical pathways in biological samples (Sansone, Melchiorre et al. Sansone et al. 2013). For the first time, we identified sapienic acid in Caco-2 cancer cells, and, after its in vitro supplementation, we followed up its metabolic transformation to 8cis-C18:1 and 5cis, 8cis-C18:2 (sebaleic acid) (Scanferlato et al. 2019) – all components of the n-10 fatty acid family. It is worth noting that sebaleic acid (5cis,8cis 18:2) is a positional isomer of linoleic acid (9cis, 12cis C18:2) but, differently from this essential fatty acid, can be de novo synthetized by cancer cells. In the same work, by application of Laurdan two-photon microscopy, fluidity was found to be different in cells under supplementation of the two positional isomers, palmitoleic and sapienic acids.

Recently, the n-10 fatty acid metabolism was followed in two prostate cancer cell lines (PC3 and LNCaP) and for the first time also in their released exosomes, which are crucial for metastatic development. We found that the total content of n-10 fatty acids reached 9–13% in cells and in exosomes (Ferreri et al. 2020). Also, the sapienic/palmitoleic ratio resulted high in these prostate cell lines, showing that the increased enzymatic activity of delta-6 versus delta-9 desaturase on palmitic acid can be used as a diagnostic signature in cancer metabolism.

We are currently applying our methodology in clinical trials for biomarker C16 isomer detection in patients, improving current knowledge in cancer correlated to fatty acid metabolism and lipid phenotypes and seeking innovations in membrane-based diagnostics (Ferreri et al. 2020b).

Applications to Other Diseases or Conditions

Membrane Lipidomics Applied to Overweight and Obese Cohorts

Lipids play a decisive role in obesity due to both their structural and molecular signaling functions. However, it is still difficult to find the fatty acid profiles used to determine the lipid metabolism in overweight or obese patients. It is instead very clear that the mature RBC membrane FA profile will allow physicians and nutritionists to know the metabolic and nutritional status of the individuals and obtain the molecular characterization of fat accumulation. To this end, Jauregibeitia et al. (2020) carried out a study in a population of normal-weight, overweight, and obese children to define the RBC membrane FA profile that characterizes obese children and to establish its relationship with metabolism and dietary habits, in order to be able to design precise nutritional strategies. Results impacted on the nutritional directions, highlighting the need to not only increase dietary sources rich in omega-3 fats but also, while increasing such foods, reduce foods containing ω-6, as well as the need for a higher intake of foods rich in MUFA fats to reduce the SFA/MUFA ratio. In 2021, another observational study was carried out to establish the respective differences in RBC membrane FA profile between adults and children with obesity, in order to establish specific and personalized nutritional recommendations according to the differences observed by age, and those derived from the metabolism of obesity itself (Jauregibeitia

et al. 2021). The characterization of RBC FA membrane profiles in children compared to the adult population revealed very different lipid profiles. Children with obesity presented higher LA, DGLA, and total omega-6 values, along with lower DHA and total omega-3 values, compared to adults with obesity. At the same time, children with obesity presented lower levels of palmitic acid (C16:0) and a lower value of the de novo lipogenic index (16:0/18:2) compared to adults with obesity.

Later a third study by Jauregibeitia et al. (Jauregibeitia et al. 2021) examined a subgroup of obese children displaying a similar lipid profile to normal-weight children by statistical clustering techniques. The analysis of the RBC membrane FA profile as a biomarker of inflammation allowed us to generate knowledge that contributes to the molecular characterization of the so-called metabolically healthy obesity (MHO). At the same time, the absence of an inflammatory profile in this group requires different nutritional recommendations tailored to their metabolic needs for effective interventions. RBC membrane lipidomics can be also applied to characterize metabolically obese normal weight (MONW), as it has been done with MHO individuals, since RBC FA profile is considered a good biomarker to define inflammatory profiles. Therefore, studying MHO and MONW individuals could provide important insights into the inter-relationships between inflammation, metabolic health, and obesity (Phillips and Perry 2013).

Although some intervention studies have already attempted to demonstrate the effect of diets rich in MUFA and ω-3, the use of lipidomics allows us to identify the baseline level and needs at the level of individual FA, so that the design of the strategy will be much more precise and will ensure the success of the intervention. However, a larger number of interventional clinical trials are needed to demonstrate the efficacy of a lipidomic-based intervention.

To re-establish the optimal composition of the RBC membrane FA profile, an adequate nutritional strategy must be established, comprised not only of an optimal diet adapted to specific metabolic needs, but in many cases, it must also be accompanied by the use of fatty acid-based supplements, depending on the precise needs of the subjects.

From a precision nutrition point of view, knowledge of the RBC membrane FA profile of individuals with obesity, together with the integration of other molecular parameters, dietary habits, preferences, and eating behavior, allows us to understand their relationships with obesity metabolism and to propose future nutritional intervention strategies, which may be effective in the long term, and reverse the increase in prevalence of obesity.

Mini-Dictionary of Terms

1. Fatty acid profile: the combination of fatty acids as quality and quantity of saturated, monounsaturated, and polyunsaturated fatty acids (SFA, MUFA, and PUFA) that are able to represent the molecular composition of a compartment, tissue, or organism.
2. Membrane lipidome: the diversity of lipids that form the membrane compartment.

3. Omega-3 index: the sum of the omega-3 fatty acids EPA and DHA, expressed as percentages of a fatty acid cohort present in the red blood cell membrane.
4. PUFA balance: the ratio between the omega-3 content EPA and DHA (expressed in percentage of fatty acid cohort present in cell membrane) and the total PUFA (omega-3 EPA + DHA/total PUFA).
5. Membrane homeostasis: characteristic property of cell membrane that is due to the membrane lipid composition, including the processes of exchange and remodeling which occur along cell life, keeping constant all structural and functional features.
6. Membrane fatty acid cohort: the group of saturated and unsaturated fatty acids (SFA, MUFA, and PUFA) that represent the diversity of the fatty acid families present in the cell membrane phospholipids can be used as representative of the status of the fatty acid pool in other tissues.
7. Membrane lipid therapy: therapeutical strategy based on lipid formulations that can be incorporated in membrane phospholipids after supplementation and are able to make clinical improvements in different diseases.

Key Facts of Fatty Acids as Biomarkers in Nutrition

Key Facts of Membrane Fatty Acids

- No cell can exist without membrane.
- Membrane composition must have a balanced presence of saturated, monounsaturated, and polyunsaturated fatty acids for the regular structure and functions of the membrane itself.
- Fatty acids constitute a lipid pool in the organisms, and in cells, that come from diet and metabolism.
- If there is insufficient intake of polyunsaturated fatty acids, the membrane composition is not balanced and has an impact on cell structure and functions.
- The membrane fatty acid analysis is very useful to evidence the deficit of PUFA intakes or of PUFA transformations in the body.
- Membrane fatty acid cohort is formed by ten fatty acids representative of saturated, monounsaturated, and polyunsaturated fatty acids.
- The membrane fatty acid cohort is a comprehensive biomarker panel for nutrition and metabolism.

Key Facts of Fatty Acid Analysis

- The analytical methodology for membrane fatty acid analysis uses the red blood cell as representative and reporter cell for the whole organism conditions.
- The red blood cell has a lifetime of 120 days and its fatty acid content represents the stabilized contributions from metabolic and nutritional status of the individual.

- Red blood cells exchange their lipids with circulating lipoproteins and tissues; therefore, its fatty acid pool can represent the availability of the fatty acids for all tissues.
- The isolation of mature red blood cell allows for obtaining information on the stabilized dietary and metabolic conditions.
- The methodology for isolation of cell membrane from blood sample and analysis of the fatty acid content is now automatized and accredited according to the ISO/EIC:17025 regulation.
- Gas chromatography is the gold standard for the separation and identification of fatty acids, distinguishing positional and geometrical isomers.

Key Facts of Membrane Fatty Acids Used in Prevention

- The membrane fatty acid cohort of ten fatty acids can be used to characterize the molecular status of the individuals regarding lipid dietary intakes and metabolic transformations.
- Evidence of fatty acid unbalance can be obtained from the membrane lipidomic analysis.
- The appropriate intervention in terms of dietary and supplementation directions can be given to patients after examining the membrane fatty acid profile.
- The fatty acid unbalance of membrane profiles can be revealed before it can create a pathological condition; therefore, it is a tool for prevention strategies.
- The membrane fatty acid profile can evidence the presence of an altered omega-6/omega-3 ratio which is a sign of pro-inflammatory condition.
- The intervention for an altered omega-6/omega-3 ratio requires both dietary and supplementation strategies.

Summary Points

- The use of membrane lipidomic profile evidences the effectiveness of fatty acid intakes, especially related to polyunsaturated fatty acids, which are essential fatty acids.
- The quantity and quality of fatty acids in membranes must respect the characteristic of each tissue and can evidence the lack or the excess of specific fatty acids, such as arachidonic acid (marker of inflammation) or EPA-DHA deficit (markers of poor omega-3 dietary intakes).
- Red blood cells are the most numerous cells formed every day and use the lipid pools present in the organism ($<70\%$ of the cells formed every day); thus, from the analysis of their cell membranes, it can be evidenced if the lipid pool contains defects.
- The RBC membrane profile can be used as a comprehensive biomarker to examine the efficiency of dietary and metabolic conditions through the balance

among the fatty acid families, through specific indexes such as omega-6/omega-3 ratio and SFA/MUFA ratio.

- The RBC membrane profile is nowadays well known for the omega-6 and omega-3 levels which are expressed by indexes such as omega-3 cardiovascular risk index (EPA + DHA) and they have specific interval determined on populations.

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Assessing Pyruvate Carboxylase Flux as a Mechanistic Biological Marker in Fasting

21

Jun Chen and Jae Mo Park

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Abstract

Pyruvate carboxylase (PC) is an enzyme that catalyzes pyruvate carboxylation for the formation of a tricarboxylic acid (TCA) cycle intermediate, oxaloacetate. It plays a major role in the regulation of gluconeogenesis and replenishment of the TCA cycle for biosynthesis. In particular, fasting promotes gluconeogenesis from pyruvate, increasing PC flux. PC is under complex regulation by hormones, substates, and cofactors, in response to nutrient changes. Therefore, metabolic changes associated with PC need to be understood in the context of nutritional and physiological conditions. This chapter discusses currently available methods, from conventional approaches to state-of-the-art noninvasive in vivo imaging techniques, used in human and animal models to assess PC activity, particularly in fasting condition. Quantification of PC activity through in vitro or ex vivo isotopomer analysis and monitoring PC in vivo using hyperpolarized substrates are reviewed.

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Keywords

Pyruvate carboxylase · Gluconeogenesis · Fasting · Anaplerosis · NMR · Mass spectrometry · Flux · Isotopomer · Biomarker · Dynamic nuclear polarization

Abbreviations

Acetyl-CoA	Acetyl coenzyme A
ALT	Alanine aminotransferase
DNP	Dynamic nuclear polarization
HP	Hyperpolarized
IV	Intravenous
LDH	Lactate dehydrogenase
LPCKO	Liver-specific knockout of *Pcx* gene
MS	Mass spectrometry
NAFLD	Nonalcoholic fatty liver disease
NMR	Nuclear magnetic resonance
PC	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
T_1	Spin-lattice relaxation times
TBI	Traumatic brain injury
TCA cycle	Tricarboxylic acid cycle

Introduction

Glucose, the major fuel for mammalian cells, is metabolized through glycolysis into the end product, pyruvate. Pyruvate can be utilized via several metabolic pathways, depending on the tissue microenvironment, nutrient state, and cell type (Fig. 1). For instance, oxygen concentration affects the fate of pyruvate. During anaerobic respiration, pyruvate is primarily reduced into lactate in the cytoplasm by lactate dehydrogenase (LDH). In aerobic condition, pyruvate can be further metabolized in the mitochondria. Before the 1950s, decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA) was considered the only pathway to enter the tricarboxylic acid (TCA) cycle. In 1952, alternative pathway entering the TCA cycle at oxaloacetate via pyruvate carboxylation was considered by Strisower et al. (1952) and later became a part of the model of mitochondrial TCA cycle and gluconeogenesis (Weinman et al. 1957; Katz 1985). Pyruvate enters the TCA cycle via two major enzymes that regulate pyruvate decarboxylation and carboxylation. The balance between the pathways depends on the cell type. In non-gluconeogenic cells such as cardiomyocytes, pyruvate is dominantly converted to acetyl-CoA by pyruvate dehydrogenase (PDH) and enters the TCA cycle for ATP production. In gluconeogenic cells like hepatocytes where pyruvate carboxylase (PC) is highly expressed, more than 80% of the pyruvate is

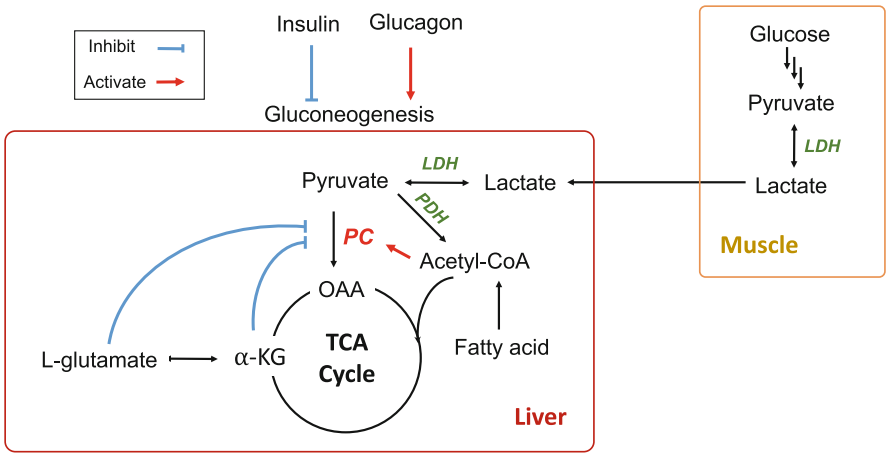

Fig. 1 Regulation of pyruvate carboxylase activity. Pyruvate carboxylase (PC) is the main regulatory enzyme of gluconeogenesis in the liver and kidney cortex, converting lactate that is exported from muscle Cori cycle. PC is regulated positively by glucagon and negatively by insulin. PC activity is also regulated by allosteric activation of acetyl-CoA, whereas selective TCA intermediates such as α-ketoglutarate or metabolites that interact with the TCA cycle (e.g., L-glutamate) are allosteric inhibitors of PC.

carboxylated into oxaloacetate for gluconeogenesis by PC in an Mg^{2+} ATP-dependent way (Utter and Keech 1960).

Since PC is in the mitochondria, the cytoplasmic pyruvate must be transported to mitochondria to be converted into oxaloacetate. Mitochondrial oxaloacetate is reduced to malate, exported to the cytosol, and oxidized back to oxaloacetate in a process called malate shuttle, for gluconeogenesis to take place in the cytosol. The cytosolic phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the irreversible conversion of oxaloacetate into phosphoenolpyruvate (PEP). PEP then goes through the reverse steps of glycolysis, plus the rate-limiting enzyme of gluconeogenesis, fructose-1,6-biphosphatase, to generate the end product glucose and complete gluconeogenesis.

PC is the main regulatory enzyme of gluconeogenesis and is positively regulated by glucagon and glucocorticoids and negatively regulated by insulin (Pilkis et al. 1988). PC activity is also regulated by allosteric activation of acetyl-CoA, which accumulates through excessive fatty acid oxidation or through cataplerosis (Adina-Zada et al. 2012). On the other hand, selective TCA cycle intermediates (e.g., α-ketoglutarate) or metabolites that interact with the TCA cycle (e.g., L-glutamate) serve as allosteric inhibitors of PC (Scrutton and White 1974). Conversely, inhibition of PC suppresses gluconeogenesis (Bahl et al. 1997). In addition to gluconeogenesis, PC also has a role in anaplerosis to replenish TCA cycle intermediates to maintain biosynthesis (Cappel et al. 2019). For example, 33–50% of the glucose entering astrocytes goes through PC for anaplerosis purpose (Weber and Barros 2015). PC is expressed in almost all human tissues and especially high in the liver and kidney

cortex (Wexler et al. 1994). In this chapter, we will focus on the metabolic role of PC in response to fasting and the methods to assess PC flux and activity.

PC Flux During Fasting

The liver is the major organ to maintain glucose homeostasis and, therefore, is sensitive to the nutritional state. The kidney is the only other organ that can generate sufficient glucose into the circulation and contributes to approximately 20% of the glucose released during prolonged fasting (Meyer and Gerich 2000). In the first few hours of fasting, the liver produces and releases glucose mainly from glycogen (Hellerstein et al. 1997). For longer fasting, gluconeogenesis dominates glycogenolysis to sustain the glucose level for metabolic demands (Hellerstein et al. 1997). Hormonal regulation plays a key role for prolonged fasting; glucagon level is upregulated to stimulate the gluconeogenesis process using pyruvate, lactate, and free amino acids (Marliss et al. 1970). Consequently, the expression level and activity of PC increase in the liver and renal cortex in response to long fasting to support the increased need for gluconeogenesis (Salto et al. 1996). Furthermore, hepatic PC sustains TCA cycle intermediates for biosynthesis and redox balance during fasting. For instance, oxidative stress, inflammatory response, and significant alteration in amino acid metabolism were observed in PC knockout mice after overnight fasting due to depleted TCA cycle intermediates and elevated NAD^+/NADH ratio (Fig. 2) (Cappel et al. 2019).

Direct Assessment of PC: Gene Expression, Protein Level, and Colorimetric Assays

Many studies have extrapolated changes in PC from the mRNA level or protein level of PC in tissue extracts (Kumashiro et al. 2013; Salto et al. 1996). The methods are useful for understanding the PC enzyme level. However, the PC enzyme level does not necessarily reflect PC enzyme activity. Other factors such as activators, inhibitors, cofactors, and post-translational modification also affect enzyme activity. Thus, any conclusions drawn about PC from protein level or gene level need to be validated with in vivo flux analysis under similar experimental conditions.

PC activity can be measured with a continuous colorimetric assay kit from tissue extract to compare the rate of reaction under different nutrient condition with various genetic backgrounds (Cao et al. 2016). However, PC enzyme activity assay is done in vitro provided with excessive substrates and cofactors, which, therefore, cannot represent the complicated in vivo physiological regulation by substrate concentration, cofactor availability, inhibitors and activators, redox state of the cell, and hormonal regulation. Additional drawback of the colorimetric assay is that the assay is based on NAD^+/NADH redox reaction, which can be triggered by other enzymes in the extract, therefore often giving an overestimation of PC activity.

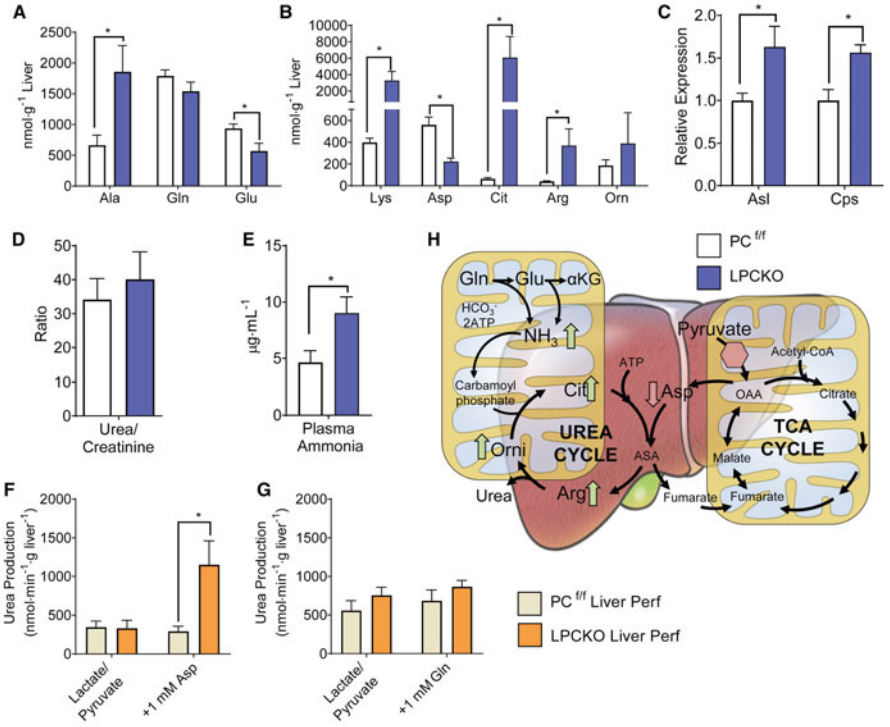

Fig. 2 Disrupted hepatic amino acid catabolism after overnight fasting in mice with liver-specific pyruvate carboxylase knockout mice. (**a**) Concentrations of alanine, glutamine, and glutamate as determined by mass spectrometry in snap-frozen livers from overnight fasted mice. (**b**) Concentrations of urea cycle intermediates and related amino acids. (**c**) Expression of genes whose products contribute to urea cycle function normalized to cyclophilin B. (**d**) Ratio of urea to creatinine in urine collected from ad-lib-fed mice. (**e**) Concentration of ammonia in the plasma of overnight fasted mice. (**f**) Rate of urea production in liver perfusion from 18 h fasted mice using perfusate with lactate/pyruvate or 1 mM aspartate supplementation. (**g**) Rate of urea production in liver perfusion from overnight fasted mice using perfusate with lactate/pyruvate or 1 mM glutamate supplementation. (**h**) Schematic illustrating the effects of liver-specific knockout of *Pcx* gene (LPCKO) on hepatic urea cycle. Data expressed as mean ± SEM. $*p < 0.05$ by Student's t-test. (Adapted from Cappel et al. (2019))

Metabolomics Approach: Isotopomer Analysis

The complexity of PC-related cellular metabolism requires system-level analysis, and, thus, it is important to be assessed in vivo. Early studies measured hepatic gluconeogenesis in fasting humans in vivo using ^{13}C NMR by monitoring glycogen concentration over a long fasting period (e.g., 68 h in Rothman et al. (1991)). This type of intrinsic ^{13}C NMR method estimates the net rate of gluconeogenesis but cannot assess PC-specific flux. Over the past few decades, flux analysis with ^{13}C-labelled, ^{14}C radioactively labelled, or deuterated isotopomer using nuclear

magnetic resonance (NMR) and mass spectrometry (MS) has been the most recognized way to study metabolic pathways (Cappel et al. 2019). The isotopomer is given to the subject with intravenous (IV) infusion until the steady state is reached; then the samples are collected and freeze-clamped immediately to preserve the metabolomics profile. It is important for the isotopic tracer infusion time to be long enough to reach steady state, which requires approximately three to five times the half-life of glucose or more than 3 h in human for gluconeogenesis study (Allick et al. 2006). This is because the enrichment of labelled products will increase over time as the unlabelled gluconeogenic precursors are consumed. If the data were collected before reaching the steady state when the entire circulating glucose pool is turned over, the labelled products will only reflect partial PC activity.

Glucose is a natural choice for tracing the fate of glucose. [U-$^{13}C_6$]Glucose infusion can provide information about glucose absorption (M+6 glucose), gluconeogenesis, and TCA cycle activity (Fig. 3). Upon infusion, [U-$^{13}C_6$]glucose is converted to [U-$^{13}C_3$]pyruvate in tissue. [U-$^{13}C_3$]Pyruvate is transported to the liver as [U-$^{13}C_3$]lactate or [U-$^{13}C_3$]alanine, which are converted back to [U-$^{13}C_3$]pyruvate and subsequently into [^{13}C]glucose. During this process known as Cori cycle (Waterhouse and Keilson 1969), PC in the liver converts [U-$^{13}C_3$]pyruvate into [2,3,4-$^{13}C_3$]oxaloacetate, which will exchange with malate, resulting in [1,2,3-$^{13}C_3$]oxaloacetate because of scrambling through fumarate. The triple labelled oxaloacetates enter the gluconeogenic pathway and generate [1,2,3-$^{13}C_3$]glucose and [4,5,6-$^{13}C_3$]glucose. On the other hand, the oxaloacetates can also go through the TCA cycle and become [2,3-$^{13}C_2$]oxaloacetate, which will be converted into [1,2-$^{13}C_2$]glucose and [5,6-$^{13}C_2$]glucose. Thus, [U-$^{13}C_6$]glucose infusion not only provides flux information of gluconeogenesis but also helps in estimating the balance of pyruvate entering gluconeogenesis and the TCA cycle by comparing [1,2,3-$^{13}C_3$]glucose and [4,5,6-$^{13}C_3$]glucose versus [1,2-$^{13}C_2$]glucose and [5,6-$^{13}C_2$]glucose. Regardless of the multiple models that quantify gluconeogenic flux with various correction factors and equations, the estimated fraction of gluconeogenesis from [U-$^{13}C_6$]glucose infusion is consistently calculated at 45–60% in healthy adults after overnight fasting (Haymond and Sunehag 2000; Hellerstein et al. 1997).

However, such infusion of glucose can result in the perturbation of nutrition state as it increases plasma glucose level and requires cautious monitoring blood glucose level when measuring PC flux during fasting. Direct precursors of oxaloacetate such as pyruvate, lactate, and alanine were also used as alternative tracers to assess PC activity and gluconeogenesis in the liver (Beylot et al. 1995; Jenssen et al. 1990; Consoli et al. 1990; Cheng et al. 2011) and kidney (Jans and Kinne 1991). [2,3-^{13}C] Pyruvate is proven as a useful tracer to compare PDH and PC activity as it is converted into [4,5-^{13}C]glutamate through PDH and [2,3-$^{13}C_2$]glutamate through PC (Jin et al. 2016a). Infused lactate and alanine are instantaneously converted to pyruvate via LDH and alanine aminotransferase (ALT), respectively, as they are in rapid exchange with pyruvate. Labelled lactate was found to be a better tracer than other substrates for assessing hepatic gluconeogenesis, as its labelling pattern is not affected by TCA cycle activity (Beylot et al. 1995). In addition, labelled glycerol and glycerate were also reported to trace PC activity as they can enter the glycolysis

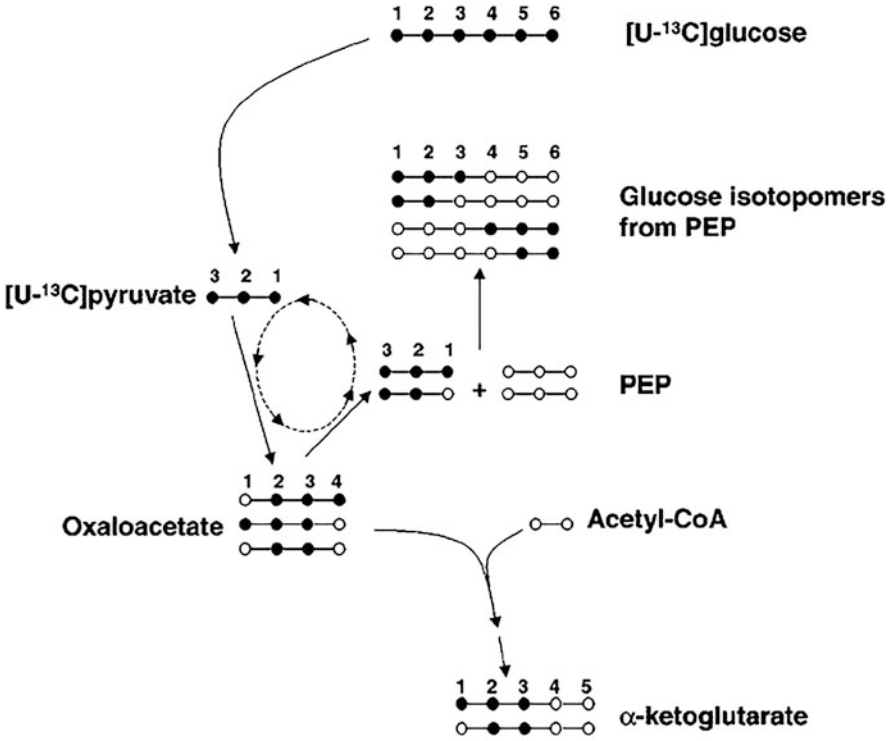

Fig. 3 Labelling pattern for [U-$^{13}C_6$]glucose infusion.. [U-$^{13}C_6$]glucose enters the glycolysis pathway to generate [U-$^{13}C_3$]pyruvate and its equivalents [U-$^{13}C_3$]lactate and [U-$^{13}C_3$]alanine. PC converts [U-$^{13}C_3$]pyruvate into [2,3,4-$^{13}C_3$]oxaloacetate and [1,2,3-$^{13}C_3$]oxaloacetate. Both triple labelled oxaloacetates lose a labelled carbon during TCA cycle and form [2,3-$^{13}C_2$]oxaloacetate. The phosphoenolpyruvate carboxykinase (PEPCK) converts labelled oxaloacetates into [U-$^{13}C_3$]phosphoenolpyruvate (PEP) and [2,3-$^{13}C_2$]PEP. Going through the gluconeogenesis pathway, [U-$^{13}C_3$]PEP becomes [1,2,3-$^{13}C_3$]glucose and [4,5,6-$^{13}C_3$]glucose, while [2,3-$^{13}C_2$]PEP is converted to [1,2-$^{13}C_2$]glucose and [5,6-$^{13}C_2$]glucose. (Adapted from Perdigoto et al. (2003))

pathway and be converted into labelled pyruvate (Jin et al. 2016b; Chen et al. 2021b). However, these tracers still have compromised accuracy of assessing PC activity as the pool size is not constant and changes with time during the infusion. Likewise, the equilibration rate between cytosolic and mitochondrial compartments can be sensitive to the long infusion, hampering accurate PC quantification.

In Vivo Assessment of PC Activity Using ^{13}C MR Spectroscopy

The abovementioned methods require tissue extraction and can not measure the real-time PC activity. Moreover, it was reported that isolated liver cannot duplicate in vivo liver metabolism precisely as it is sustained by the non-physiological buffer (Jin et al. 2016a). Thus, noninvasive in vivo approaches are preferred for metabolic

assessment. A common in vivo method to trace PC activity is to directly acquire ^{13}C spectrum using MRS with steady-state infusion of ^{13}C-labelled substrate. For instance, flux analysis of PC-regulated metabolism was demonstrated in the human liver in vivo by measuring the production rate of [2-^{13}C]glutamate from infused ^{13}C-acetate (Petersen et al. 2016). In vivo application of ^{13}C MRS is, however, challenged by compromised signal-to-noise ratio (SNR) of the labelled metabolites due to the poor MR sensitivity of ^{13}C nuclei, resulting in limited spectral and spatial information despite hours of infusion time. The limited MR sensitivity and typically long (tens of seconds) longitudinal relaxation times of ^{13}C-metabolites require a long acquisition time and, thus, are clinically inadequate.

There are largely two approaches to overcome the SNR problem. The first approach is to acquire MR data in high-field environment as the MR signal is proportional to the magnetic field strength (B_0). Strong B_0 improves not only SNR but also the chemical shift dispersion, allowing better peak separation between different ^{13}C-labelled products. Researchers have been using ultrahigh field MRI to study pyruvate carboxylation and anaplerosis in the brain in combination with ^{13}C-labelled glucose infusion (Fig. 4) (Cheshkov et al. 2017). Alternative approach is the application of dissolution dynamic nuclear polarization (DNP) to ^{13}C-labelled compound. Dissolution DNP is a relatively new technique that can amplify ^{13}C MR signal to more than 10,000–100,000-folds as compared to its thermal signal measured at conventional MRI magnets (e.g., 3 T) (Ardenkjaer-Larsen et al. 2003). Although hyperpolarization in ^{13}C via DNP needs to be performed in extremely low temperature (~1 K) and high magnetic field, its rapid dissolution process can maintain the amplified MR signals in liquid state, allowing the administration of the hyperpolarized (HP) solution to living organism for in vivo MRI. Immediately after an IV injection of HP solution, metabolic products via enzymatic processes are visible.

However, the polarized signals are transient as most spin-lattice relaxation times (T_1) are several minutes or seconds. In addition, unlike conventional ^{13}C MRS studies, HP studies are performed with a bolus injection of solution that contains highly concentrated (20–160 mM for animals and ~250 mM for humans) HP ^{13}C substrates. Therefore, HP studies acquire snapshots of in vivo metabolism with transient, non-recoverable labelled tracer.

HP [1-^{13}C]pyruvate is the most studied HP substrate by far for its pivotal position that connects glycolysis to several pathways via LDH, PDH, ALT, and PC. Its investigational use for human imaging study was approved by the United States Food and Drug Administration (Kurhanewicz et al. 2019). Several studies used HP [1-^{13}C]pyruvate to investigate PC activity in fed/fasted conditions (Merritt et al. 2011; Jin et al. 2016a). While [^{13}C]HCO_3^- can be produced via multiple pathways such as PC-PEPCK, PC-TCA, and PDH (Merritt et al. 2011; Jin et al. 2016a; Chen et al. 2021a), detection of [1-^{13}C]malate, [4-^{13}C]malate, [1-^{13}C]aspartate, and [4-^{13}C]aspartate are considered as PC-specific products (Fig. 5) (Lee et al. 2013). However, resolving malate and aspartate peaks from large [1-^{13}C]pyruvate hydrate and [1-^{13}C]alanine peaks in the adjacent resonance is challenging in conventional 3 T or lower magnetic field due to limited chemical shift dispersion (Jin et al. 2016a). Moreover, HP [1-^{13}C]pyruvate is a suboptimal probe to study PC since bolus injection of pyruvate may alter the NAD^+/NADH ratio and shunt the pyruvate

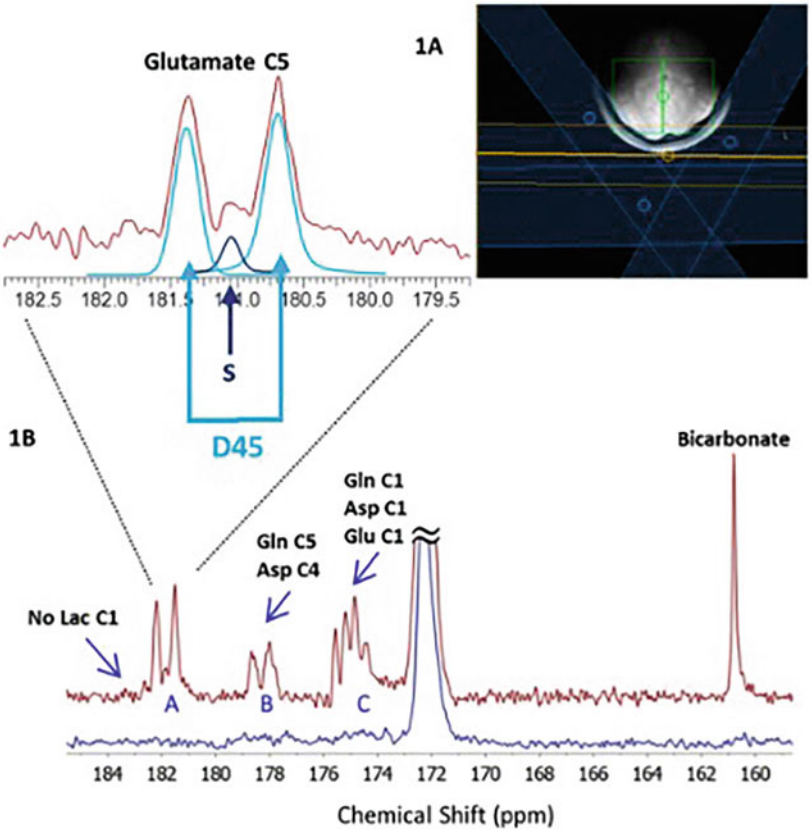

Fig. 4 Detection of pyruvate carboxylase and pyruvate dehydrogenase pathways using [U-$^{13}C_6$] glucose in the human brain at 7 T under steady-state conditions. (**a**) Proton image showing the occipital brain area investigated by ^{13}C MRS. (**b**) Representative post-infusion proton-coupled ^{13}C spectrum (in red) of a healthy subject showing the detection of infused [U-^{13}C]glucose products; same experiment with no glucose infusion (in blue). (Zoom-in) The glutamate C5 singlet, S, and doublet D45 fitting. (Adapted from Cheshkov et al. (2017))

toward PDH. As a PC-favorable substrate, HP lactate was proposed (Chen et al. 2021a). Since plasma and tissue concentration of lactate is five to ten times higher than pyruvate, HP [1-^{13}C]lactate measures PC activity without disrupting the redox. Indeed, [1-^{13}C]aspartate could be resolved at 3 T, and its appearance was sensitive to nutrient condition (Fig. 6).

Complication with Other Pathological Conditions

In this chapter, we reviewed PC as a biomarker for fasting and methods to measure the PC flux. It is noteworthy that PC is also sensitive to other conditions and diseases, including but not limited to nonalcoholic fatty liver disease (NAFLD),

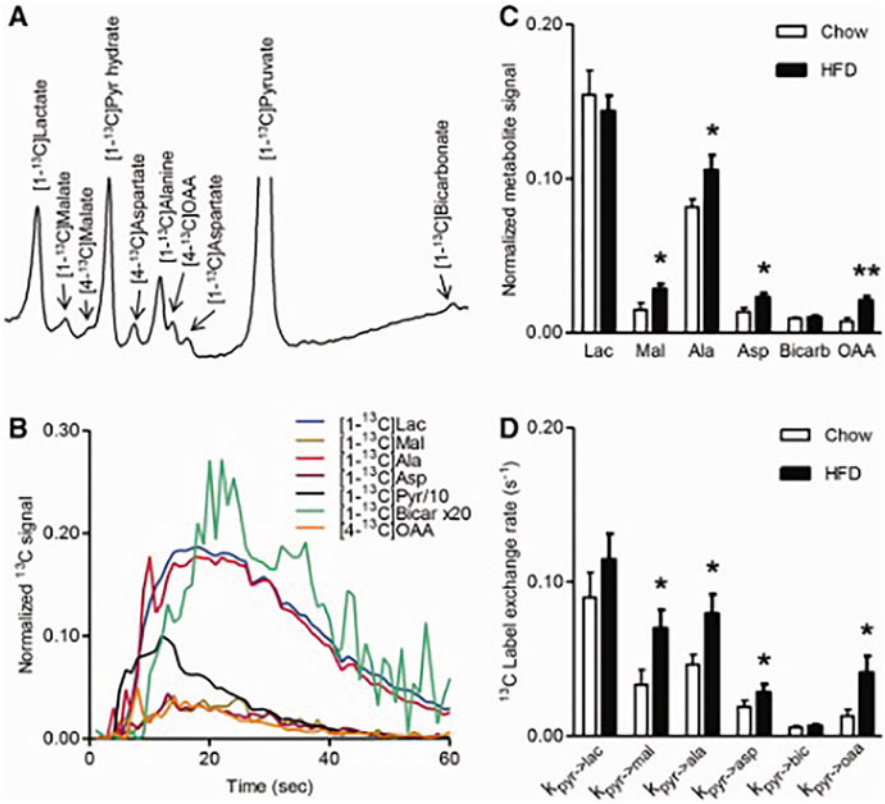

Fig. 5 Measurement of in vivo pyruvate anaplerosis in mice by ^{13}C MRS with a bolus injection of hyperpolarized [1-^{13}C]pyruvate. (**a**) Hyperpolarized ^{13}C spectra in fatty liver. Metabolites that were detected include PC-specific products such as [1-^{13}C]oxaloacetate, [1-^{13}C]malate, [4-^{13}C]malate, [1-^{13}C]aspartate, and [4-^{13}C]aspartate in addition to products via PDH (e.g., [^{13}C]bicarbonate). (**b**) Representative time course depicting the simultaneous production of downstream metabolites upon infusion of hyperpolarized [1-^{13}C]pyruvate. (**c**) Normalized metabolite signal comparison between mice fed on Chow and high-fat diet. (**d**) Corresponding ^{13}C label exchange rates ($N = 8$/group). Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. OAA, oxaloacetate; HFD, high-fat diet. (Adapted from Lee et al. (2013))

type II diabetes, and traumatic brain injury (TBI). Insulin suppresses lipolysis and decreases the level of hepatic acetyl-CoA, which is an allosteric activator for PC, thereby inhibiting PC activity (Perry et al. 2015). In NAFLD and type II diabetes with insulin resistance, insulin fails to regulate the PC activity, resulting in increased hepatic gluconeogenesis and lipid synthesis (Perry et al. 2015). Preliminary data in rodent models showed that PC knockout is beneficial for blood glucose level, liver gluconeogenesis, liver fatty acid synthesis, and liver fatty acid oxidation (Kumashiro et al. 2013). Therefore, PC is believed to be one of the main biomarkers for glucose homeostasis in insulin resistance and provides a potential therapeutic target.

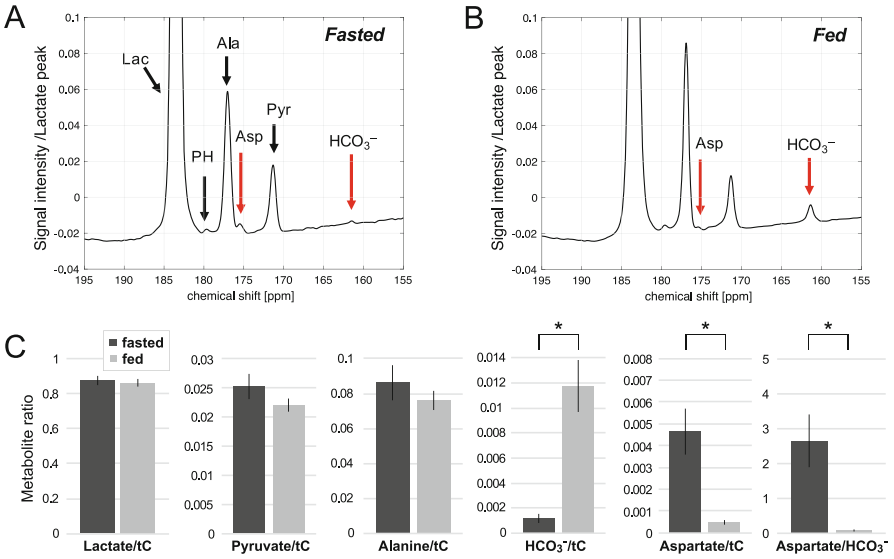

Fig. 6 Effects of nutritional status on in vivo hepatic metabolism using hyperpolarized [1-^{13}C]-l-lactate. Time-averaged spectra of 60 mM hyperpolarized [1-^{13}C]-l-lactate in the liver of (**a**) fasted and (**b**) fed rats. The spectrum is normalized to the maximum [1-^{13}C]lactate peak intensity. (**c**) Comparison of lactate signals and products, normalized by the total hyperpolarized carbon (tC) signals in the fasted and fed groups. *Statistical significance between the groups ($P < 0.01$). Ala, alanine; Asp, aspartate; HCO_3^-, bicarbonate; Lac, lactate; PH, pyruvate hydrate; Pyr, pyruvate; tC, total carbon. (Adapted from Chen et al. (2021a))

The brain accounts for 20% of the energy utilization in human using glucose as the major energy source. Patients with TBI have a broad spectrum of dysregulated glucose metabolism, with both hyper- and hypometabolism commonly seen, depending on the severity and timing of the injury (Koenig and Dulla 2018). Animal models of TBI revealed that hypometabolism in the brain is related to increased PC/PDH function as glucose is more used for anaplerosis with increased PC for regeneration rather than energy production (Bartnik et al. 2007). The challenge to treat TBI in a timely manner is extensive, but PC could serve as a predictive biomarker for injury progression or recovery monitoring.

Mini-dictionary of Terms

- **Dynamic nuclear polarization**. A technique that amplifies MR signal of targeted nuclei such as carbon-13 or nitrogen-15. In combination with rapid dissolution process and MRI, DNP can be used for imaging in vivo metabolism.
- **Gluconeogenesis**. A metabolic pathway that synthesizes glucose from non-carbohydrate substrates such as lactate. This pathway is considered as one of the key pathways in carbohydrate metabolism.

- **Isotopomer analysis**. A quantitative technique that measures metabolic steps by differentiating the labelling patterns of metabolites. NMR and mass spectrometry are commonly used for isotopomer analysis.
- **Pyruvate carboxylase**. A mitochondrial enzyme that converts pyruvate and bicarbonate to oxaloacetate. Pyruvate carboxylase is an entry enzymatic step in gluconeogenesis.
- **Pyruvate dehydrogenase**. Alternative enzyme for pyruvate to enter mitochondria via decarboxylation. Pyruvate is converted to acetyl-CoA, which can be subsequently metabolized in the tricarboxylic acid cycle.

Key Facts of Pyruvate Carboxylase

Pyruvate carboxylase is a mitochondrial enzyme that converts pyruvate to oxaloacetate.

Pyruvate carboxylation was first considered as an alternative pathway for entering the tricarboxylic acid cycle in 1952.

Pyruvate carboxylase is the main regulatory enzyme of gluconeogenesis.

Pyruvate carboxylase activity is regulated by glucagon, glucocorticoids, insulin, and allosteric activation of acetyl-CoA.

Pyruvate carboxylase is expressed in almost all human tissues and especially high in the liver and kidney cortex.

The expression level and activity of pyruvate carboxylase increase in the liver and renal cortex in fasting condition.

Pyruvate carboxylase activity can be assessed with colorimetric assay kit in vitro, isotopomer analysis using NMR or mass spectrometry ex vivo, or ^{13}C MR spectroscopy using hyperpolarized ^{13}C-substrates in vivo.

Summary Points

- Pyruvate carboxylase activity and its expression level increase in the liver and renal cortex in response to long fasting to support the increased need for gluconeogenesis.
- In vitro enzyme assay of pyruvate carboxylase does not represent the complicated in vivo physiological regulation such as substrate concentration, cofactor availability, and cellular redox state.
- Flux analysis with labelled isotopomers can be used to study pyruvate carboxylase using nuclear magnetic resonance and mass spectrometry ex vivo.
- Dissolution dynamic nuclear polarization of carbon-13-labelled substrates can assess pyruvate carboxylation and gluconeogenesis in vivo.
- Hyperpolarized [1-^{13}C]pyruvate detects real-time changes of products via pyruvate carboxylase and pyruvate dehydrogenase in response to fed/fasted conditions.

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Biological Markers of Plant Phenolic Compounds Intake

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Abstract

Phenolic compounds (PC) are a diverse class of phytochemicals that are thought to be among the major responsible for the association between high intake of fruits and vegetables and low risk of chronic diseases. Despite promising protective effects in vitro and in animal models, human studies associating PC intake to beneficial health effects have showed more variable results. The accurate assessment of dietary intake of the different classes of PC is a critical step in epidemiological or intervention studies and biomarkers of PC intake are an alternative to the traditional dietary assessment tools. This chapter focuses on the relation between PC intake and the concentration of PC and their metabolites in body fluids (plasma and urine). The biotransformation of PC during digestive process and their kinetics following exposure, as well as the methods used for estimating dietary intake of PC are also discussed.

Keywords

Plasma · Urine · Biotransformation · Nuclear magnetic resonance spectroscopy · Mass spectrometry · Microbiota · Digestive process · Flavonoids · Tannins · Phenolic acids · Stilbenes · Lignans · Coumarins

Abbreviations

BBB	Blood-brain barrier
CBG	Cytosolic β-glucosidase
FFQ	Food frequency questionaries
GC	Gas chromatography
HepG2	Hepatocellular carcinoma cell line
HRMAS	High-resolution magic angle spinning
LC	Liquid chromatography
LPH	Lactase-phlorizin hydrolase
MCT	Monocarboxylic acid transporter
MS	Mass spectrometry analyzer
NMR	Nuclear magnetic resonance spectroscopy
ODMA	O-desmethylangolensin
PC	Phenolic compounds
SGLT1	Sodium-glucose-linked transporter 1
UDP	Uridine diphosphate

Introduction

Fruits and vegetables have been usually associated with a decreased risk of non-transmissible chronic diseases, such as cancer and cardiovascular diseases. Phenolic compounds (PC) are the most likely bioactive compounds associated to these health benefits. These non-nutrient phytochemicals are widely found in the plant kingdom and comprise more than 8000 structures characterized by one or more hydroxyl groups attached to at least one benzene ring (Crozier et al. 2009).

In order to establish firm evidence for the health effects of PC consumption, it is essential to have accurate quantitative information regarding their dietary intake. Despite *in vitro* and animal experiments have provided strong positive evidence associating PC to numerous health benefits, evidence from human studies is not strong enough. Most epidemiological studies use food frequency questionnaires to determine the dietary intake, which results in imprecision (Picó et al. 2019).

Biological markers of PC exposure consist of one or more biochemical moieties being measured in an accessible fluid or tissue to provide a semiquantitative index of the exposure to individual food constituents (Spencer et al. 2008). Biomarkers in epidemiological studies have to be indicators of exposure and must be robust, sensitive to changes, specific to the dietary source, and biologically relevant (Spencer et al. 2008). Before a particular PC or its metabolite can be used as a sensitive and accurate biomarker, a number of factors must be taken in account. Firstly, a full understanding of the metabolism of PC in human subjects is required in order to select credible biomarkers. Secondly, it is important to understand the time–response relationship between PC intake and the appearance of the biomarker in biological fluids. Thirdly, the precise dose–response relationship between the intake of a

specific PC and the appearance of its biomarker is essential. Finally, one must also have an understanding of the extent to which certain physiological and environmental factors affect the rate of PC metabolism in human subjects.

The relationship between dietary intake of PC and resulting concentrations of PC biomarkers in body fluids is highly complex. Usually, the bioavailability of PC is low due to their poor solubility and rapid transformations. Moreover, most PC are metabolized by human gut microbiota generating products that may be absorbed in the gut and display a beneficial role in several organs (Augusti et al. 2021). To date, the concentration of some PC (daidzein, genistein, glycitein, and hydroxytyrosol) in urine showed high correlation with the intake dose, revealing good sensitivity and robustness as biomarkers of intake throughout different studies. On the other hand, weaker correlations of urinary anthocyanins with the intake dose suggest that they are currently less suitable as biomarkers of PC intake (Pérez-Jiménez et al. 2010).

This chapter summarizes current evidence regarding the relationship between the concentration of PC or their metabolites in body fluids (plasma and urine) and the intake of PC. The biotransformation of PC during digestive process and their kinetics following exposure, as well as the methods used for estimating dietary intake of PC were also discussed.

Classification and Structure of Major Dietary PC

PC are products of the secondary metabolism of plants, providing essential functions for their survival (Augusti et al. 2021). Structurally, PC comprise a benzene ring bearing one or more hydroxyl substituents, and range from simple molecules to complex polymers (Tsao, 2010). According to their chemical structure, they can be divided into several classes that may differ significantly in stability, bioavailability, and bioactivity (Tsao 2010): phenolic acids, tannins, lignans, flavonoids, stilbenes, coumarins, and curcuminoids (Fig. 1). PC can also be divided into free, esterified, and insoluble-bound forms, depending on whether they occur in the free form or are covalently bound to other molecules such as fatty acids (soluble esters) or insoluble macromolecules (insoluble-bound PC). Most insoluble-bound PC are bound to cell

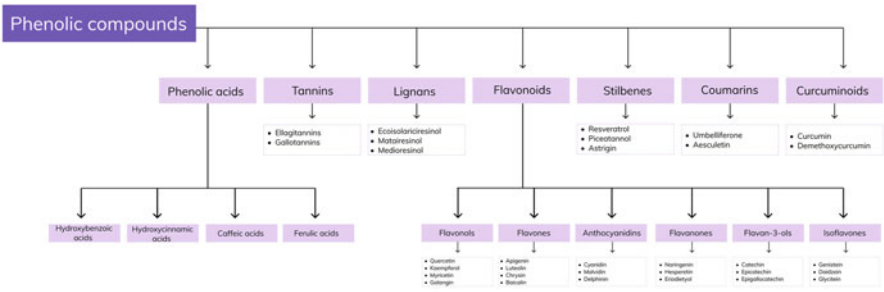

Fig. 1 Chemical structure of main PC classes. Classification and representative compounds of the main classes of PC

wall substances including pectin, cellulose, arabionoxylan, and structural proteins and account for relatively large amount (20–60% in vegetable, fruits, and legume/seeds) compared to the soluble PC in foods. Given that the usual analytical methods for the measurement of the total contents of PC do not consider the insoluble-bound forms, the content of PC in foods should be higher than those described in nutritional tables (Shahidi and Yeo 2016).

Phenolic Acids

Phenolic acids are the simplest class of PC and their basic structure contain one phenolic ring and a carboxylic acid function. According to their carbon skeleton, they can be subdivided into hydroxybenzoic acids (derived from benzoic acid) or hydroxycinnamic acids (derived from cinnamic acid), the latter one comprising the most common compounds, such as caffeic and ferulic acids (Albuquerque et al. 2021). Fruits and vegetables contain mostly free phenolic acids that are readily solubilized during digestion and in the solvents used for the quantitative analysis of PC in food sources. On the other hand, phenolic acids from grains and seeds are often found covalently bound to complex structural carbohydrates, which do not solubilize during digestion or in the hydroalcoholic solvents currently used for PC analysis (Tsao 2010).

Flavonoids

Flavonoids are a PC group mainly consisting of a benzopyrone ring bearing phenolic or polyphenolic groups at different positions. The structural complexity of flavonoids has led to their subclassification into flavonols, flavones, flavanones, flavan-3-ols (including their oligomeric and polymeric forms, proanthocyanidins), isoflavones, and anthocyanins. This classification considers their chemical structure, the degree of unsaturation, and the oxidation of carbon ring (Cassidy and Minihane 2017). In plants, most flavonoids exist as glycosides, although their basic structures are aglycones. The biological activities of these compounds, including their antioxidant activity, depend on the structural difference and the glycosylation patterns. Also, the number of hydroxyl groups, configuration, and substitution are responsible for their antioxidant capacity (Albuquerque et al. 2021).

Tannins

Tannins are a unique group of PC with molecular weights between 500 and 30,000 Da widely distributed in almost all plant foods and beverages. Condensed and hydrolysable tannins are the two major groups of these bioactive compounds, but complex tannins contain structural elements of both groups (Serrano et al. 2009). Hydrolysable tannins are formed by simple phenols, e.g., ellagic and gallic acids,

bound to carbohydrate moieties, while condensed tannins are formed by condensing two or more than 200 monomers of flavan-3-ol units.

Stilbenes

Stilbenes represent a class of compounds with a common 1,2-diphenylethylene backbone that have shown extraordinary potential in the biomedical field. As the most well-known example, resveratrol proved to have antiaging and other important health effects (Andrei et al. 2019). Stilbenes are characterized by two benzene rings connected by a double bond, which depending on its configuration, divide the class into the isomers Z and E that usually have different biological activities.

Lignans

Lignans are a class of PC that usually contain a core scaffold formed by two or more phenylpropanoid units linked by the central carbons of the side chains (Cui et al. 2020). They are usually present as dimers, but some of them are trimers or tetramers. Most plant lignans are in the free state, while some of them can combine with sugar to form glycosides and other derivatives. The monomers forming lignans are cinnamic acid, cinnamyl alcohol, propenyl benzene, and allyl benzene. Lignans can be grouped in classical lignans when the molecular linkage of monomers occurs between positions β-β′ and in neolignans, when the main structural units are coupled in any other way (Cui et al. 2020).

Coumarins

Coumarins are 1,2-benzopyrones that consist of a benzene ring linked to a pyrone ring. Based on modifications of this core, coumarins can be classified into complex and simple coumarins. Complex coumarins are produced by the addition of heterocyclic compounds on the basic coumarin core and are further classified into furanocoumarins, pyranocoumarins, phenylcoumarins, dihydrofurocoumarins, and biscoumarins. Simple coumarins includes scopolin, scopoletin, esculin, esculetin, umbelliferone, fraxetin, and sideretin (Stringlis et al. 2019).

Curcuminoids

Curcuminoids are a class of PC found in turmeric (*Curcuma longa*), a common spice used in the preparation of curries in India and other Asian countries because of its flavor and color. Among curcuminoids, curcumin accounts for approximately 77%, while demethoxycurcumin accounts for 17% and bisdemethoxycurcumin accounts for 3–6% (Kotha and Luthria 2019), all belonging to the diarylheptanoid family.

Chemically, curcumin consists of two similar aromatic rings each with o-methoxy phenolic groups, connected by linear carbon chain, having an α,ß-unsaturated ß-diketone moiety. While curcumin is the most studied curcuminoid, it has low bioavailability. Thus, structures without the methoxy group on the benzene ring of the parent structure, such as demethoxycurcumin, has greater stability and has been associated to health benefits (Hatamipour et al. 2019).

Biotransformation of PC During Digestive Process

Food digestion comprises a complex combination of chemical, biochemical, and mechanical processes that operate to disintegrate food into small particles and convert macromolecular nutrients into their basic units that will be available for absorption. The process starts in the mouth where chewing reduces the size of solid foods, which are mixed with saliva reaching pH values between 5 and 7. The pH of digesta rapidly drops out to values between 1 and 3, when it reaches the stomach, being subsequently neutralized to pH values between 6 and 7.5 at the small intestine (Sensoy 2021). Although human enzymes are responsible for the biotransformation of food constituents up to the small intestine, they do not directly act on PC. However, PC may be released upon food matrix disintegration due to the digestion of carbohydrates, proteins, and lipids. Moreover, pH changes along the gastrointestinal tract play a major role in the transformation of PC up to the small intestine (Quatrin et al. 2020; da Silva et al. 2021).

Most dietary PC (90% of intake) will not be solubilized and absorbed up to the small intestine and will therefore reach the colon (Kawabata, Yoshioka and Terao 2019), where gut microbiota triggers the deconjugation of glycoside, glucuronide, and organic acid moieties. Then, PC-derived aglycones are released and subsequently cleaved by fission of heterocyclic and aromatic rings, and undergo dihydroxylation, decarboxylation, demethylation, reduction, and isomerization of alkene moieties (Cortés-Martín et al., 2020). Some of these biotransformation pathways have been elucidated (Fig. 2). This extensive transformation by gut microbiota generates small molecular weight compounds (Fig. 2) that usually have higher absorption rate and therefore can reach higher plasma levels than their parent PC. In addition, many of these microbial-derived PC metabolites have bioactive effects and are actually the major responsible for the systemic biological effects of dietary PC (Cortés-Martín et al. 2020).

The other face of the interplay between PC and gut microbiota is the reshape of the former one by dietary phenolics in a prebiotic-like effect (Cortés-Martín et al. 2020). These changes in microbiota composition may affect its catabolic activity, inducing further changes in the bioavailability and profile of microbial-derived PC metabolites, which in turn affects the bioactivity of dietary PC (Mena et al. 2019). In addition, as the composition of gut microbiota is remarkably affected by diet composition, fecal bacteria have been recently suggested as biomarkers for predicting specific foods and food group intake (Shinn et al. 2021).

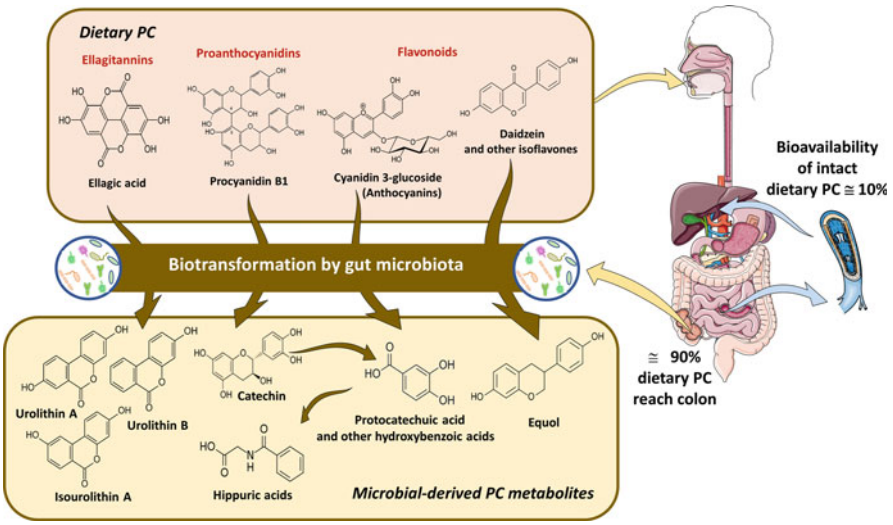

Fig. 2 Overview of the major metabolic pathways in the biotransformation of dietary PC by the colonic gut microbiota. This figure shows the biotransformation of PC in their metabolites by colonic microbiota

This section will address the potential of microbial-derived PC metabolites to be used as biomarkers of PC intake. The great structural diversity of PC leads to distinct biotransformation pathways during digestion, which are being unveiled by recent advances in metabolomic analysis and will be further addressed according to the class of PC.

Anthocyanins and Other Flavonoids

Anthocyanins are quite stable at the acidic gastric pH but are readily converted into hemiketal, chalcone, and quinoidal forms at the intestinal pH values (Quatrin et al. 2020). Delayed neutralization of gastric chyme by intestinal fluid seems to increase anthocyanin stability and bioavailability (da Silva et al. 2021). The absorption of anthocyanins in forms that preserve their aglycone moieties is estimated to be approximately 1% of total ingested amount (Zhong et al. 2017). Most anthocyanins will reach the colon where they are deglycosylated, and undergo opening of the C-ring to form phenolic acids and aldehydes (Catalkaya et al. 2020). Cyanidin glucosides are primarily transformed into protocatechuic acid (also known as 3,4-dihydroxybenzoic acid) (Vitaglione et al. 2007). In fact, protocatechuic acid and other hydroxybenzoic acids are also the major microbial metabolites of other anthocyanins (pelargonidin, peonidin, malvidin, delphinidin, and petunidin glycosides) and even of non-anthocyanin flavonoids (Catalkaya et al. 2020; Cortés-Martín et al. 2020). After human intake of cranberry juice, plasma levels of protocatechuic acid have been shown to be higher than its parent PC (McKay et al. 2015).

Interestingly, protocatechuic acid has been demonstrated to be the actual responsible for some biological effects of anthocyanins, such as the antiatherogenic effects of dietary cyanidin-3-glucoside in a mice model of atherosclerosis (Wang et al. 2012).

As anthocyanins, most of other dietary flavonoids usually occur as O- or C-glycosides, which will also be deconjugated by gut colonic bacteria (Cortés-Martín et al. 2020). The aglycone moieties released share a common heterocyclic pyrone C-ring that links benzene rings A and B. The C-ring can be opened through demethylation and dihydroxylation reactions generating simpler PC derived from rings A and B with hydroxyl or methyl ester radicals. Flavonols, flavanones, and flavones share some common transformation products. The flavanone hesperidin is converted into its aglycone form, hesperitin, that can be further catabolized into phenylpropionic, phenylacetic, and hippuric acids. Hydroxyphenylpropionic acid has been described as the major fermentation product of naringenin and kaempferol, and the final product of eriodictyol and quercetin (Catalkaya et al. 2020).

Hydrolyzable Tannins

Gut bacteria can hydrolyze the ester bonds of gallo- and ellagitannins (Cortés-Martín et al. 2020). Gallic acid can undergo decarboxylation and dihydroxylation reactions. Urolithins are major metabolites of ellagic acid–related PC (Kawabata, Yoshioka and Terao 2019; Quatrin et al. 2020), being formed upon dihydroxylation and intramolecular condensation. Urolithin A (dihydroxy-urolithin) and urolithin B (hydroxy-urolithin) are produced by most population, while type C and D urolithin production is less frequent (Catalkaya et al. 2020). There is also a group of population that do not produce urolithins, likely due to the absence of some *Gordonibacter* bacteria (Catalkaya et al. 2020). The distinct profile in the production of urolithins is remarkably affected by aging and allowed the identification of three ellagic acid metabotypes in the population. Metabotypes are described as specific microbial-metabolic phenotypes that result in qualitative differences among individuals (metabolite producers vs. nonproducers) rather than a metabolite production gradient in the population (Cortés-Martín et al. 2020). Metabotype A is characterized by the production of only urolithin A, whereas metabotype B produces urolithin B and isourolithin A in addition to urolithin A, and metabotype 0 are those individuals that do not produce urolithins (Cortés-Martín et al. 2020). Despite the variability in the metabolic profile between individuals, urinary urolithin A, along with the phase II metabolite di-methyl ellagic acid, have been recently used as biomarkers of ellagitannin intake from black raspberry food products (Roberts et al. 2020).

Flavan-3-Ols

Different from other flavonoids, flavan-3-ols, which is the major class of native flavonoids in human diet, usually do not occur as glycosides but may occur as monomeric or polymeric compounds (Zamora-Ros et al. 2013, 2016). Studies in

ileostomized humans indicate that more than 70% of flavan-3-ols will reach the colon after the intake of green tea (Stalmach et al. 2010).

Polymeric flavan-3-ols, such as proanthocyanidins, which are also known as condensed tannins, are first converted into their monomeric units (catechins). Subsequent microbial catabolism converts catechins into hydroxyphenyl-γ-valerolactones, which are thereafter sequentially converted into the following phenolic acids: hydroxyphenylvaleric, hydroxyphenylpropionic, hydroxyphenylacetic, hydroxybenzoic, and hippuric acids (Appeldoorn et al. 2009). Although many catabolic routes involved in these transformations remain to be elucidated, it is clear that the major gut metabolites of flavan-3-ols are hydroxyphenyl-γ-valerolactones and, to a lesser extent, their derived hydroxyphenyl valeric acids (Mena et al. 2019). These compounds could be used as potential biomarkers of flavan-3-ols intake because they have high absorption rate and reach relevant circulating concentrations (Ottaviani et al. 2018, 2019; Mena et al. 2019). Nevertheless, methodological difficulties in the accurate chromatographic quantification of polymeric flavan-3-ols in food matrices poses a hindrance to establish the relationship between flavan-3-ols intake and the biological levels of their metabolites (Mena et al. 2019). In addition, the lag time between the intake of flavan-3-ols and the assessment of hydroxyphenyl-γ-valerolactones or their derived hydroxyphenyl valeric acids should also be considered. These flavan-3-ol metabolites are intermediates that will be further catabolized into other phenolic acids, which can be also generated from the catabolism of many other PC (Cortés-Martín et al. 2020).

All these microbial-derived flavan-3-ol metabolites can be absorbed and metabolized by phase II enzymes producing conjugated derivatives that are subsequently eliminated in the urine (Mena et al. 2019). The lack of commercial analytical standards of microbial-derived flavan-3-ols metabolites, especially for the conjugated compounds, limits their assessment in biological fluids to tentative identification and quantification.

Isoflavones

Isoflavones such as daidzein is metabolized by gut microbiota generating equol and O-desmethylangolensin (ODMA), whereas genistein generates 5-hydroxy-equol and 6′-hydroxy-ODMA (Cortés-Martín et al. 2020). Two metabotypes have been already identified for daidzein, the equol- and ODMA-producer individuals (Mayo, Vázquez and Flórez 2019). These metabotypes are independent from each other and their population distribution has been shown to be associated to dietary patterns, sociodemographic characteristics, race, and ethnicity (Cortés-Martín et al. 2020).

Phenolic Acids

Most phenolic acids escape absorption in the small intestine and will reach the colon intact. Simple hydroxybenzoic acids, such as gallic acid, ellagic acid, protocatechuic

acid, and 4-hydroxybenzoic acids, are usually found at low concentrations in foods, but they may appear as constituents of complex PC, such as the hydrolysable tannins or as metabolites of hydrolysable tannins and flavonoids (Cortés-Martín et al. 2020). Their metabolic fate during digestion has been already discussed in previous sections.

Hydroxycinnamic acids, such as p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid, are commonly found in foods in the free form or esterifying sugars and organic acids (quinic, tartaric, and malic). After hydrolysis by bacterial esterases, hydroxycinnamic acids are released and metabolized to phenylpropionic acids and subsequently to hydroxybenzoic acids (Cortés-Martín et al. 2020).

Hippuric acid, which can be formed from the conversion of the quinic acid moiety of PC by gut microbiota but also from the hepatic metabolism of benzoic acids, is the most common and abundant urinary and plasma metabolite detected after the intake of various foods rich in PC (Catalkaya et al. 2020). Although hippuric acid is also formed during the metabolism of aromatic amino acids, its urinary levels have been shown to be consistently increased after the intake of tea (Clifford et al. 2000; Daykin et al. 2005), cocoa (Rios et al. 2003), vegetables, and fruits (Krupp et al. 2012). Thus, urinary levels of hippuric acid, along with 3,4-dihydroxyphenylacetic acid and simple hydroxycinnamic acids, have been proposed as candidate biomarkers for the intake of a PC-rich diet in a cross-sectional study of free-living individuals (Yang et al. 2019).

Lignans

Only 7% of the ingested lignans have been shown to be absorbed in the small intestine after the intake of a cereal source containing secoisolariciol, matairesinol, lariciresinol, pinoresinol, syringaresinol, and medioresinol by catheterized pigs that were used as a model for humans (Bolvig et al. 2016). Colonic fermentation plays a key role in the bioavailability and bioactivity of lignans that are catabolized by gut microbiota into enterodiol and enterolactone, also known as enterolignans. In fact, about 40% of the ingested cereal lignans were excreted as enterolignans in the urine (Bolvig et al. 2016). Diverse bacterial species are involved in the complex metabolic pathway that starts with the deglycosylation of lignans and is followed by reduction, demethylation, dihydroxylation, and lactonization reactions to generate the final metabolites, enterolignans (Cortés-Martín et al. 2020).

Kinetics of PC After Dietary Intake

Absorption

Dietary PC are usually chemical structures of high complexity with ester bonds that may appear as polymerized and glycosylated forms, which prevent their direct absorption. The absorption of some PC seems to start in the oral cavity after the

aglycone release by the action of glycosidases from buccal microbiota (Hussain et al. 2019). Anthocyanins have been detected in the plasma 5 min after the contact with oral tissues (Kamonpatana et al. 2014). Around 83% of the total amount of anthocyanins and 46% of non-anthocyanin PC from jaboticaba peel (*Myrciaria trunciflora*) powder were solubilized during the gastric phase of digestion (Quatrin et al. 2020). Phenolic acids and anthocyanins can be absorbed in their free forms in the stomach, via passive diffusion or monocarboxylic acid transporter (MCT)-mediated transport system and bilitranslocase proteins (Bohn 2014). However, most PC undergo hydrolysis and deconjugation reactions in the gastric compartment or remain intact until reaching the intestine (Williamson and Clifford 2017).

The small intestine and colon are considered the main sites for PC absorption (Matuschek et al. 2006). Both the chemical structure of the phenolic moiety and any attached chemical groups define whether the PC is absorbed in the small intestine or colon (Williamson and Clifford 2017). In the small intestine, PCs are subjected to the action of digestive enzymes such as cytosolic β-glucosidase (CBG), lactase-phlorizin hydrolase (LPH), and esterases, which promote the cleavage and release of aglycones from the remaining food matrix (Bohn 2014). Phenolic acids and aglycones of low molecular weight are absorbed by the enterocytes through passive diffusion. Conversely, compounds of high molecular weight and complex chemical structure use cotransporters mediated by sodium-glucose-linked transporters 1 (SGLT1) and MCT (Hussain et al. 2019). However, the physicochemical characteristics of the intestine can contribute for the decomposition of anthocyanins, even before their absorption takes place (Grgić et al. 2020). For instance, only 10% of anthocyanins from jaboticaba peel were recovered after the intestinal phase of digestion, while recovery indexes between 52% and 134% were found for non-anthocyanin compounds (Quatrin et al. 2020). The intestinal pH around 7 favors the transformation of anthocyanins into a chalcone pseudo-basis that can be cleaved at the C-ring and originates phenolic acids (Bohn 2014). Possibly, this fact contributes to the low bioavailability of anthocyanins in plasma. Furthermore, intestinal microbiota also plays a prominent role in the colonic absorption of PC. Anthocyanins and other polymeric PC are subject to the action of microbial esterases that are not produced by mammalian cell. Such esterases convert polymeric PC into simple phenolic acids and phenolic metabolites, capable of being absorbed (Williamson and Clifford 2017).

Distribution

Most data on the tissue distribution of PC come from animal studies. The distribution of PC and their metabolites depends on the dose administered, solubility, need for tissue-specific carriers, and tissue functionality (Carecho et al. 2020). Once absorbed, the PC and their metabolites depart from the small intestine to the liver, via the portal circulation, to undergo a series of phase I and II biotransformation reactions and are distributed into the bloodstream mostly bound to proteins, especially albumin (Cao et al. 2019). Due to their low absorption, only a small portion of PC and their metabolites are deposited in tissues (Bohn 2014), mainly in those

tissues where they have been metabolized. However, they can also reach specific target tissues such as pancreas, brain, heart, and spleen (Velderrain-Rodríguez et al. 2014).

Anthocyanins appear to be more abundant in gastrointestinal tissues such as the stomach, jejunum, ileum, and colon in rats (Kalt 2019). Even so, anthocyanin content was greatest in the kidneys, liver, heart, lungs, and brain of rodents (Sandoval-Ramírez et al. 2018). Curiously, the treatment time seems to influence in the bioavailability profiles of anthocyanins in animal tissues. Anthocyanin metabolites are predominant in short-term experiments, while the parent anthocyanin in long-term experiments (Sandoval-Ramírez et al. 2018). However, this does not seem like a definitive rule. A study where rats consumed a single dose of 400 mg of the bilberry extract Bilberon/kg of weight body demonstrated major amounts of parent anthocyanins in tissues and fluids (e.g., urine, feces, and bile) when compared to anthocyanin metabolites (Ichiyanagi et al. 2006).

Liver tissue was the second largest site of deposition of phase II metabolites of catechin and epicatechin and the first for naringenin-glucuronides and sulfates or even products of hydroxylation/methylation of anthocyanins (Margalef et al. 2015; Lin et al. 2014; Fornasaro et al. 2016). These data are expected since the main flavonoid metabolism occurs in this tissue (Manach et al. 2004). The incorporation of hydrophilic conjugates allows these compounds to be mainly eliminated in the urine. Thus, it is not surprising that phase II metabolites of catechin and quercetin were found at the highest amount in the kidneys (Margalef et al. 2015; Yang et al. 2016).

The distribution to the brain is very small, as not all PC or metabolites can cross the blood-brain barrier (BBB) (Margalef et al. 2015). Factors such as chemical structure, polarity, metabolism by phase I and phase II enzymes, intestinal microbiota, and pathways used to access the brain can affect the permeability of the BBB (Carecho, Carregosa and dos Santos, 2020). Nevertheless, animal studies revealed that intact forms of anthocyanins (cyanidin-3-glucoside) (Fornasaro et al. 2016) and metabolites such as naringenin-sulfates, quercetin-3-O-β-glucuronide, or methylated derivatives of catechin and epicatechin (Lin et al. 2014; Yang et al. 2016; Margalef et al. 2015) were found in brain tissue or cerebrospinal fluid. Moreover, dietary PC have recently been found in the cerebrospinal fluid of patients with neurological disorders, showing that dietary compounds can also cross the BBB in humans (Grabska-Kobylecka et al. 2020).

Metabolization

Following absorption of aglycones and the formation of their conjugated derivatives via phase II reactions in the small intestine, PC metabolites rapidly reach the liver by transferring via portal blood (Donovan et al. 2006). In the liver, both conjugated and unmetabolized aglycones can undergo phase I and phase II metabolism (Velderrain-Rodríguez et al. 2014), with additional conversions and enterohepatic recirculation which may result in the re-excretion of compounds into gut lumen through biliary route (Crozier et al. 2010; Donovan et al. 2006).

Concerning hepatic phase I metabolism, flavonoids may be biotransformed via oxidation or O-demethylation reactions by cytochrome P450 monooxygenases. However, phase I metabolism contributes to minor metabolites of most flavonoids and other PC, as these compounds undergo conjugation reactions faster than oxidation (Manach et al. 2004).

Intestinal and hepatic phase II reactions of PC consist in the conjugation of methyl, sulfate, and/or glucuronic acid moieties to the hydroxyl groups of PC. These structural modifications detoxify and facilitates the biliary and urinary elimination of PC metabolites (Manach et al. 2004). However, this biotransformation decreases the bioefficacy of PC in relation to their parent aglycones (Scalbert and Williamson 2000).

Catechol-O-methyl transferase activity produces O-methylated metabolites by transferring a methyl group from S-adenosyl-L-methionine to the 3′ position (or to a lesser extent to the 4′ position) of PC having a catechol moiety. 3-O-methylated derivatives have been reported for quercetin, catechin, caffeic acid, luteolin, and cyanidin (Manach et al. 2004). A substantial amount of a 4'-O-methylated product was reported for 4′-methylepigallocatechin in human plasma following ingestion of tea (Lee et al. 2002; Meng et al. 2001).

Sulphotransferases generate O-sulfates mainly in the liver by catalyzing the transfer of a sulfate moiety from 3′-phosphoadenosine-5′-phosphosulfate to a hydroxyl group on PC, which can contain multiple possible conjugation site depending on the compound and enzyme isoform. Phenolic acids such as caffeic and dihydrocaffeic acids are both sulfated in 3-OH and 4-OH whereas the flavonoids daidzein and genistein are sulfated in 7-OH and 4'-OH and resveratrol in 3 and 4'positions (Wu et al. 2011).

UDP-glucuronosyl transferases localized in the endoplasmic reticulum is highly expressed in intestine, liver, and kidney and produce O-glucuronides by catalyzing the transfer of a glucuronic acid from UDP-glucuronic acid to PC. Interestingly, glucuronidation of PC first occurs in the enterocytes before further conjugation in the liver (Crespy et al. 2001; Manach et al. 2004).

In general, the profile of phase II metabolites of PC is complex and the preference for the type of conjugation reaction may vary even among compounds within the same chemical class (Velderrain-Rodríguez et al. 2014). For hydroxycinnamic acids, it was shown that methylation, glucuronidation, and sulfation were the preferential pathways for caffeic acid metabolism, whereas ferulic acid was only glucuronidated and chlorogenic acid showed null metabolism in HepG2 cells (Mateos et al. 2006). The ingested dose must also be considered, since a shift from sulfation toward glucuronidation occurs when the ingested dose of PC increases (Manach et al. 2004). Conversely, quercetin metabolism in HepG2 cells shifts from glucuronidation toward sulfation when methylation reaction is inhibited (O'Leary et al. 2003). Several other factors also influence on the balance between sulfation and glucuronidation, such as stereochemistry, polymorphism of phase II enzymes, metabolism site, species, sex, and food deprivation (Velderrain-Rodríguez et al. 2014). Saturation of the conjugation reactions can also occur, as observed in rats exposed to high doses and rats given an acute supply of PC by gavage (Piskula and Terao 1998).

Regardless of the type of phase II reactions, the production of conjugated metabolites from PC is a highly efficient process so that absorbed PC circulate

mainly on the conjugated forms. Accordingly, aglycones are either not found in blood or are presented in low concentrations following consumption of nutritional doses (Velderrain-Rodríguez et al. 2014) except for anthocyanins and tea catechins (Lee et al. 2002). Unfortunately, circulating conjugated metabolites have been identified for only a few PC. 3-O-glucuronide, 3'-O-methylquercetin 3-O-glucuronide, and quercetin 3'-O-sulfate were the major conjugated metabolites from quercetin in human plasma following consumption of onions containing quercetin glucosides (Day et al. 2001), even though the presence of sulfated quercetin was not further corroborated (Wittig et al. 2001).

Excretion

Unmetabolized PC, their phase II metabolites, and bacterial derivatives are excreted through urine and feces. Most phase II metabolites undergo first-pass metabolism and are transferred to the large intestine where they will be subjected to colonic microbiota metabolism (Velderrain-Rodríguez et al. 2014). For large, extensively conjugated metabolites, the biliary route is preferred. On the other hand, urinary excretion is the mainly excretion pathway for phase II metabolites (especially small conjugates), since conjugation reactions convert PC into more hydrophilic compounds (Manach et al. 2004).

Methods Used for Estimation of Dietary Intake of PC

It is difficult to quantitatively measure the benefits of consuming PC due to many variables: (1) a great diversity of PC in the diet; (2) limited data on the PC composition and content in the food databases; (3) limited understanding about the absorption and metabolism of PC; and (4) limitations in the methods for analyzing PC metabolites in biological fluids (Spencer et al. 2008; Zamora-Ros et al. 2012; Pinto and Santos 2017).

Therefore, the study of biological markers of exposure is a tool that has been used in many studies with PC (Manach et al. 2005; Mennen et al. 2006; Pérez-Jiménez et al. 2010; Clarke et al. 2021). Thus, we could have an index of consumption of PC in body fluids that could estimate the exposure of the individual food. However, this relationship between food intake and the concentration of biomarkers in body fluids is extremely complex. In this topic, we will approach the main methods used to estimate the food consumption of PC.

Dietary Assessment Methods

In nutritional studies, the intake of nutrient and non-nutrient food components is generally performed using diet assessment methods, such as diet histories, food frequency questionaries (FFQ), or diet diaries (Zamora-Ros et al. 2012; Burkholder-

Cooley et al. 2017; Pinto and Santos 2017). Subsequently, these intake data are transformed into quantitative information using food composition databases. However, several recent studies have shown the difficulty in comparing results of dietary assessment methods among different studies (Pinto and Santos 2017). This occurs due to several factors, such as the difficulties found with the participants' answers, as they are reflecting the foods they consume without underreported or overreported information. The lack of global food composition databases that comprise full composition of PC is another relevant issue. Food composition databases have scarce data on regional or local foods, on the variations in the content and composition of PC in foods grown in different regions, on the differences in the food processing and storage (Zamora-Ros et al. 2012; Burkholder-Cooley et al. 2017; Pinto and Santos 2017). Additionally, the composition and content of matrix-bound PC, which are not soluble in the conventional extractive solvents used for PC analysis, is missing from food composition databases. Finally, there are many variables to be considered when using diet assessment methods, which limits its accuracy to reflect the situation of the population at the study site and its usefulness to express the world situation.

The main methods used to assess dietary data are 24-hour diet recalls and semiquantitative FFQ (Zamora-Ros et al. 2012; Pinto and Santos 2017). The difficulty in comparing the results and the variability of results among different studies are mainly due to differences in the methods used and the use of different databases to calculate the consumption of PC. Therefore, the choice of method for collecting dietary data determines the accuracy of results.

FFQ are very useful and used by studies using a large number of individuals because they are easier and simpler, although they only reflect information on the frequency of consumption of the food in question. If we need information about the portion size, these questionnaires need adaptations and validations before use (Zamora-Ros et al. 2012; Pinto and Santos 2017). In such case, an additional problem may arise: How to standardize this portion measure? Portion sizes can be made by household measurements, which can increase the chance of errors, or they can be reported in units such as grams or even accompanied by portion size photographs. These points must be well explained and standardized in the questionnaire (Pinto and Santos 2017; Clarke et al. 2020, 2021).

The 24-hour diet recall provides more detailed information about all foods and beverages consumed during the day (Burkholder-Cooley et al. 2017). Despite being more accurate than FFQ methods, only a 24-hour recall does not represent the individual's habitual consumption. Ideally, more than one 24-hour recall should be performed on nonconsecutive days, and these recalls should be held throughout the year, because eating habits can vary a lot during the weekdays and some foods rich in PC compounds are seasonal and suffer great changes in consumption along the year (Pinto and Santos 2017).

Biomarkers

As most studies on PC intake use data from food questionnaires, biomarkers could be used to obtain more accurate measurements of tissue exposure to PC, which

consider interindividual variability in the absorption and metabolism of these compounds.

A biomarker should be quantitatively associated with the ingestion of the compound in question, that is, it must reflect differences in its consumption. Accordingly, several studies associate the ingestion of PC with biomarkers in blood and urine (Manach et al. 2005; Spencer et al. 2008; Pérez-Jiménez et al. 2010; Zamora-Ros et al. 2012; Clarke et al. 2021). Given that PC metabolism is fast, urine biomarkers are more frequently used because they have gone through all stages of metabolization. Pharmacokinetic studies revealed that most PC have half-lives between 1 and 12 h in plasma (short-term biomarkers) and between 1 and 5 days in urine (medium-term biomarkers); and long-term intake (weeks or months) markers are still lacking (Zamora-Ros et al. 2012).

Additionally, the chemical form of the PC present in food (glycosylated compounds, e.g.) and the food composition can influence the metabolism and, therefore, the time of appearance and concentration of metabolites in plasma and urine (Spencer et al. 2008).

Plasma

There is limited data regarding the precise half-lives of PC in plasma. Data suggest that these half-lives are about 2 h for anthocyanins and flavanones and 2–3 h for flavanols (Spencer et al. 2008; Zamora-Ros et al. 2012). The rapid excretion of PC is facilitated by the conjugation of aglycones or O-methylated forms to sulphate or glucuronic acid. Therefore, many studies to date have analyzed plasma PC levels 1–6 h post-intake (Manach et al. 2005; Spencer et al. 2008). The main limitation of the use of biomarkers in plasma is the wide variation in the half-life of PC when considering intestinal absorption. For example, the plasma half-life of PC absorbed in the small intestine usually ranged between 1 and 12 h. However, if we consider metabolites from colonic microbiota (e.g., equol, a colonic metabolite of daidzein), the half-life increases to >2 days (Spencer et al. 2008; Zamora-Ros et al. 2012).

According to Manach et al. (2005), the plasma concentration of total metabolites ranges from 0 to 4 μmol/L after the intake of 50 mg of aglycone equivalents. The PC that are most well absorbed in humans are isoflavones and gallic acid, followed by catechins, flavanones, quercetin glucosides and proanthocyanidins (galloylated tea catechins), and anthocyanins. Another important point is that some PC have high affinity for proteins, such as plasma albumin, which increases their elimination time. An example of this are flavonols such as quercetin, which has an elimination time of 11–28 h due to its high affinity for albumin (Spencer et al. 2008).

Thus, studies on plasma biomarkers should ideally be carried out with multiple blood samples over a 24-hour period. However, as blood collection is an invasive method, usually one collection of fasting blood is obtained, which can lead to errors and absence of the biomarker due to the metabolization process (Manach et al. 2005).

Urine

Urine biomarkers have been suggested to be more suitable than plasma to assess PC intake since absorbed metabolites can be rapidly removed from the circulation, both

by enteric tissues and by urine excretion (Spencer et al. 2008). However, the time of appearance of metabolites in urine varies widely (Mennen et al. 2008; Pérez-Jiménez et al. 2010). Thus, urine is useful to assess the intake of PC that have short to medium half-lives. Another advantage is that urine collection is a noninvasive method. However, the number of metabolites excreted via urine may not accurately reflect the quantities consumed. The urinary half-life of individual PC metabolites is highly variable and ranges from approximately 1 h to just over 1 day and can be influenced by the type and structure of foods consumed (Spencer et al. 2008; Zamora-Ros et al. 2012). Thus, most research has focused on 24-hour urine samples, whereas few studies used one-spot urine sample (Mennen et al. 2008; Pérez-Jiménez et al. 2010; Pinto and Santos 2017). Researchers should consider that 24-hour urine sampling is viable and applicable for studies with a limited number of subjects. On the other hand, for epidemiological studies involving a large number of people, 24-hour urine sampling can become a problem, and the collection of a one-spot urine sample is more coherent (Pinto and Santos 2017).

In fact, some studies have found good correlations between 24-hour and one-spot urine samples collected during a same day, indicating that a spot sample may be used to assay potential biomarkers of PC intake (Mennen et al. 2008). Additionally, studies found that many metabolites estimated in urine are strongly correlated with the consumption of PC (Mennen et al. 2008; Pérez-Jiménez et al. 2010).

Pérez-Jiménez and collaborators (Pérez-Jiménez et al. 2010) conclude that some metabolites are more promising as biomarkers because they show a great recovery in urine and a good correlation with the ingested dose: daidzein, genistein, formononetin, and glycitein. Another study shows that chlorogenic acid, m-coumaric acid, gallic acid, quercetin, isorhamnetin, kaempferol, hesperitin, naringenin, enterolactone, and enterodiol measured in spot urine samples are potentially useful biomarkers for PC intake (Mennen et al. 2008).

A meta-analysis has shown that daidzein, glycitein, enterolactone, and hydroxytyrosol had both a high recovery yield in urine and a high correlation with the intake of isoflavones, flaxseed, and olive oil, respectively. Other studies suggested that dihydrocaffeic acid-3′-O-sulphate and feruloylglycine were considered suitable urine biomarkers for coffee ingestion; conjugates of the flavanones hesperidin and naringenin for orange juice; (epi)gallocatechin, (epi)catechin, and methyl-(epi) catechin metabolites for flavan-3-ols, whereas free and conjugated forms of urolithins may be used as markers of ellagitannins (Pinto and Santos 2017).

Finally, some factors can influence the accuracy of PC metabolite analyses, such as: the stability of metabolites in biological fluids, sample preparation methods, and, essentially, the analytical method, instrument sensitivity, and availability of standards for identification (Spencer et al. 2008; Pinto and Santos 2017). In conclusion, biomarkers should not be used as the sole measure of dietary PC intake but can be used in combination with dietary assessment methods to provide more complete information about what people are consuming and the bioavailability of bioactive PC forms.

Analytical Techniques Used to Identify and Quantify PC Biomarkers

Concerning metabolomic analysis for the search of biomarkers, two analytical platforms highlight among the wide variety of techniques currently available in the scientific scenario. The analysis of several kinds of metabolites is commonly carried out by nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry analyzers (MS), these last commonly coupled to a separative technique such as liquid chromatography (LC) or gas chromatography (GC) (Scalbert et al. 2014; Gibbons et al. 2015). These analytical methodologies differ in their sensitivity, sample processing requirements, and metabolome coverage. Among these techniques, NMR is a robust and fast detection method for high abundance metabolites characterized by a high reproducibility and the potential for the identification of compounds with identical molecular weights, which could not be uniquely elucidated by other techniques. Indeed, NMR highlighted for the study of unknown compounds structures (Markley et al. 2017). NMR is an unbiased technique based on the detection of protonated compounds in the sample despite their differential physical properties. Moreover, recent advances in the field have been resulted in cryogenically cooled probes, microcoil probes, high-performance radio frequency coils, and high-field-strength superconducting magnets. All these improvements are translated into an enhanced sensitivity of NMR by several folds, which is critical for metabolomics applications (Rolin 2012; Putri et al. 2013). Furthermore, NMR is commonly known by its high interlaboratory reproducibility, which is very appreciated in broad-based analyses of high cohort metabolomic studies (Pan and Raftery 2007). Additionally, NMR via stable isotopic labeling techniques could provide a deep understanding of the mechanisms and dynamics related to various metabolic pathways (Nagana Gowda and Raftery 2015). This technique is also capable of detecting compounds that could not be ionized in MS with little or no sample preparation (Ulaszewska et al. 2019). Moreover, one of the main advantages in metabolomic studies is that NMR is a nondestructive technique, so the biological sample used for the analysis could be conserved and reanalyzed in different batches. This fact is of great importance due to the difficulty of sample collection of several biological fluids for their scarcity and invasive procedures needed. Another positive characteristic is that site-specific NMR imaging approach could be effective in the research of metabolic events in living organs (Fan and Lane 2016), whereas high-resolution magic angle spinning (HRMAS) could be used for the analysis of intact tissues (Beckonert et al. 2010). Therefore, all these aspects make NMR a good choice for being the selected analytical technique in varied multitude metabolomic studied. On the other side, NMR requires higher quantities of biological sample for their analysis and possesses lower sensitivity compared to MS (Wishart 2008).

Nevertheless, despite all the advantages above detailed of NMR, in the case of phytochemical biomarkers, MS coupled to LC or GC is extensively used in metabolomic applications. In this sense, GC-MS is suitable for the analysis of different metabolites found in vegetable or biological samples, such as amino acids, sugars, organic acids, steroids, fatty acids, and volatile metabolites analysis.

The major negative side of GC-MS analysis is the hard labor of sample treatment, consisting in a time-consuming derivatization step which could complicate quantitative approaches.

However, for the analysis of PC and their metabolites in plants and biological samples in metabolomic studies, the ideal analytical platform is LC-MS. MS is extremely sensitive, and its coupling with high-performance LC allows the separation and detection of almost 10,000 features and the identification of a wide range of chemical species. It has been reported that this platform could determine between 400 and 1500 substances depending on the metabolomic approach, targeted versus untargeted (Cajka et al. 2017). Compound identification can be made by combining retention times information with m/z along with additional analyses, such as fragmentation patterns from tandem MS, to compare against standards and spectral libraries (Sparkman et al. 2011). Additionally, their different modalities cover a wide range of compounds nature, from more polar compounds analyzed by hydrophilic interaction chromatography to neutral and nonpolar metabolites with reversed-phase chromatography. This reverse mode, using polar mobile phase (acidified water with organic solvents like methanol or acetonitrile) combined with nonpolar stationary phase (such as C-18 columns), is applied for the analysis of PC and their metabolites. Its limitation to fully characterize the complete metabolome resides in more than one single LC-MS methodology is needed. This fact, together with lower reproducibility, laborious sample treatment and certain lack of information for an undoubted spectral assignments and compounds elucidation to a high confidence level, summarizes the major bottleneck for its application in PC biomarkers discovery (Ulaszewska et al. 2019), even though some of these limitations are currently overriding (Cajka et al. 2017).

Conclusion

Current knowledge does not allow to identify a single biomarker of total PC intake. Due to the great structural diversity of PC, various candidate biomarkers are being pursued for the different classes or structure-related compounds within each PC class. The bioavailability of parent PC, which is usually low, has driven biomarker search to PC metabolites which are formed by human digestive enzymes, phase I and II enzymes, and colonic microbial enzymes.

Remarkable advances in analytical tools allowed to increase the sensitivity for the assessment of parent PC and their metabolites in biological samples but the lack of analytical standards remains a bottleneck for the accurate quantification of many PC metabolites. Some useful biomarkers have been identified for the intake of specific PC. Despite the growing interest on microbial-derived PC metabolites, the lack of specificity and the great variability in the gut microbiome/metabolic profile among population groups (Pérez-Jiménez et al. 2010) remains a relevant issue for their use as biomarkers of PC intake.

Applications to Other Diseases or Conditions

In this study, we review some useful biomarkers that have been identified for the intake of specific PC. However, the available literature does not allow pointing a single biomarker of total intake of PC. Except for studies on phytoestrogens, the available literature provides only few reports in which biomarker measurements of PC were applied. Thus, in a near future, the knowledge of biomarkers of PC intake may be useful in the risk prediction for some diseases, such as cancer, diabetes, and cardiovascular diseases.

Mini-Dictionary of Terms

- *Phenolic acids:* Class of PC containing one phenolic ring and a carboxylic acid function. The most common compounds of this class are caffeic and ferulic acids.
- *Flavonoids:* Class of PC subclassified into flavonols, flavones, flavanones, flavan-3-ols (including proanthocyanidins), isoflavones, and anthocyanins. Most flavonoids exist in plant as glycosides.
- *Tannins:* Class of PC with high molecular weight. They are divided into hydrolysable (formed by simple phenols bound to carbohydrate moieties) and condensed (two or more than 200 monomers of flavan-3-ol units condensed) tannins.
- *Stilbenes:* Class of PC characterized by two benzene rings connected by a double bond with resveratrol as its most known compound.
- *Lignans:* Class of PC with a core scaffold formed by two or more phenylpropanoid units linked by the central carbons of the side chains. The monomers forming lignans are cinnamic acid, cinnamyl alcohol, propenyl benzene, and allyl benzene.
- *Coumarins:* Class of PC with fragrant properties consisting of a benzene ring linked to the pyrone ring. This class can be divided into simple and complex coumarins.
- *Curcuminoids:* Class of PC found in turmeric (*Curcuma longa*). Curcumina is the main compound and consists of two similar aromatic rings each with o-methoxy phenolic groups, connected by linear carbon chain.

Key Facts

Key Facts of PC Digestion and Metabolism

- PC may suffer transformations during digestive processes generating new compounds.
- Most of dietary PC will not be absorbed up to the small intestine and will reach the colon, where gut microbiota triggers the catabolism of PC.
- The half-lives of PC usually range from 1 to 12 h in plasma and 1–5 days in urine.

Key Factors of Biomarkers of PC Intake

- The main methods used to assess dietary data are 24-hour diet recalls and semiquantitative food frequency questionnaires.
- Food frequency questionnaires are very useful and used by studies using a large number of individuals because they are easier and simpler, although they only reflect information on the frequency of consumption of the food in question.
- The 24-hour diet recall provides more detailed information about all foods and beverages consumed during the day, although only a 24-hour recall does not represent an individual's habitual consumption.
- The chemical form of the PC influences the metabolism and, therefore, the time of appearance and concentration of metabolites in plasma and urine.

Summary Points

- PC are associated to the protective effect of a high intake of fruits and vegetables against chronic diseases.
- PC have low bioavailability and suffer catabolism by gut microbiota.
- Food frequency questionnaires are incomplete to determine the role of PC against diseases.
- The concentration of PC or their metabolites in body fluids (plasma and urine) may be a valuable tool to determine the actual beneficial role of these compounds.

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Part IV

Genetic, Molecular, and Cellular Variables

Blood Gene Expression of Zinc Transporters as Biological Indicators of Zinc Nutrition

23

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Abstract

Zinc is an essential mineral for the growth, development, and differentiation of all types of organisms, presenting three major functions (catalytic, structural, and regulatory), and plays a relevant role in oxidative stress, immune, and inflammatory responses. Its homeostasis is maintained through mechanisms which include regulating gene expression, such as those encoding zinc transporters. There are two families of mammalian zinc transporters: the ZnT family (SLC30) and the ZIP family (SLC39). A significant variety of transporters present different expression patterns in response to zinc intake or under different nutritional statuses. Thus, understanding them may help identify subtle changes in zinc status. Furthermore, different diseases such as diabetes, cardiovascular disease, and

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Alzheimer's disease affect the expression of several transporters, being correlated with their diagnosis or prognosis. However, this is largely unexplored and constitutes a promising area to investigate for potential biomarkers to assess zinc status and its relation to diseases.

Keywords

Zinc transporters · SLC30 · ZnT · SLC39 · ZIP · Gene expression · Zinc status · Zinc homeostasis · Zinc metabolism · Alzheimer's disease · Cardiovascular diseases · Diabetes

Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	β-Amyloid peptide
CER	Cerebellum
CVD	Cardiovascular diseases
EAD	Early-stage Alzheimer's disease
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
HPG	Hippocampus/parahippocampal gyrus
IL	Interleukins
INS-1	Insulinoma cell line
IR	Insulin resistance
LAD	Late-stage Alzheimer's disease
LADA	Latent autoimmune diabetes in adults
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MCI	Mild cognitive impairment
mRNA	Messenger RNA (gene expression)
MT	Metallothionein
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor kappa B
NIA-AA	National Institute on Aging and Alzheimer's Association
PBMC	Peripheral blood mononuclear cells
PCAD	Preclinical stage of Alzheimer's disease
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
SLC	Solute carrier
SNP	Single-nucleotide polymorphisms
SOD	Superoxide dismutase enzyme
TGN	Trans-Golgi network
TMAO	Trimethylamine N-oxide
TNF-α	Tumor necrosis factor-α

VSMC	Vascular smooth muscle cell
ZIP	Zrt, Irt-like protein
ZnT	Zinc transporter

Introduction

Zinc is essential for the growth, development, and differentiation of all types of organisms and is one of the most abundant trace elements in the human body. It is found in several tissues, but its highest concentration (about 85%) is in muscle and bone tissue, and only 1% is in blood circulation (Prasad 2014).

It is estimated that zinc participates in the constitution of more than 2,700 enzymes, many of which are involved in the metabolism of carbohydrates, proteins, and lipids and the synthesis and degradation of nucleic acids. Zinc has a catalyst function in approximately 70% of these enzymes, but it can also have a structural role, act as a substrate, or act as an activity regulator (Fukada et al. 2011).

Zinc plays a relevant role in immune and inflammatory responses and oxidative stress through its catalytic, structural, and regulatory functions (Fukada et al. 2011). The regulatory functions of zinc in the inflammatory response occurs trought the role of zinc in the regulation of nuclear factor kappa B (NF-κB), via activation of A20 and peroxisome proliferator-activated receptors (PPAR). NF-κB is a highly conserved transcription factor during evolution, and its translocation to the cell nucleus regulates the expression of hundreds of genes, such as pro-inflammatory cytokines, acute-phase proteins, and adhesion molecules, among others. Thus, protein A20 indirectly inhibits the expression of cytokines with pro-inflammatory action, such as interleukins (IL) 1β, IL-6, and IL-8 and tumor necrosis factor-α (TNF-α) (Prasad 2014).

The structural function of zinc is to stabilize cell membranes since zinc deficiency is associated with increased osmotic fragility of red blood cells in humans. Some authors suggest its involvement in forming dematin, an essential protein for maintaining cell morphology, motility, and structural membrane integrity (Ryu et al. 2012).

The primary antioxidant role of zinc is to participate in the structure of superoxide dismutase 1 and 3 enzymes (SOD1, SOD3) and to preserve metallothionein (MT) concentrations since this trace element cannot directly interact with a reactive oxygen species (ROS) (Marreiro et al. 2017).

Approximately 10% of the human proteome consists of proteins potentially linked to zinc, which explains the role of zinc in DNA and RNA synthesis and proteins that preserve the stability of the genome. These actions are due to their participation in regulating and structuring proteins involved in DNA repair (Ryu et al. 2012; Fukada et al. 2011).

Zinc Metabolism and Homeostasis

Several physiological systems contribute to body zinc homeostasis under different conditions, and the gastrointestinal tract is one of the main systems responsible for this

mechanism. Homeostasis is obtained by modulating the amount of dietary zinc absorbed and the amount of endogenous zinc excreted. Regulation of urinary excretion occurs when there are extremely high or low intakes of zinc. Furthermore, tissue and cell redistribution of zinc can also favor homeostasis (Maares and Haase 2020).

The small intestine is the primary site of exogenous zinc absorption in humans, which is regulated by diffusion mechanisms and transporter-mediated processes. Active transport is saturable at high metal concentrations in the lumen of the intestine, and its efficiency is increased during low intake periods. Absorption occurs in conditions of high zinc intake through a passive diffusion mechanism without saturation. Part of the zinc in the intestinal lumen comes from pancreatic, biliary, and intestinal secretions, as well as from mucosal cell desquamation (Maares and Haase 2020).

After being absorbed, zinc is released by the enterocyte basolateral membrane transporters, passes to the mesenteric capillaries, and is directed to the portal circulation, then taken up by the liver, and distributed to other tissues (Fig. 1). Its transport in the blood is mediated by albumin and, to a lesser extent, by α-macroglobulin, transferrin, cysteine, and histidine (Maares and Haase 2020).

Zinc homeostasis is maintained through mechanisms which include regulating gene expression, such as those encoding MT and zinc transporter proteins. However, when the dietary intake of zinc is very low, the homeostatic mechanisms may be insufficient to replace the losses, resulting in a negative balance of the mineral (Maares and Haase 2020).

Zinc Transporters

Zinc transporters are membrane proteins which ensure the transport of zinc ions through the various cell structures. They are specialized in the capture, efflux, and compartmentalization of the mineral, helping to maintain intracellular and corporal homeostasis (Kambe et al. 2021).

There are two families of mammalian zinc transporters: the ZnT family (SLC30) and the ZIP family (SLC39). The ability of selective zinc binding is common to all zinc transporters, but bound zinc ions in ZnTs and ZIPs are transported in opposite directions (Fig. 2). The complementary functions of ZnTs and ZIPs stabilize the cytosolic zinc concentration around a homeostatic set point while also enriching zinc in the lumen of specific subcellular compartments to support zinc-dependent cellular processes (Kambe et al. 2021).

The ZnT family consists of ten transporter proteins which act to decrease intracellular zinc levels by transporting zinc from the cytoplasm to the lumen of organelles or the extracellular space. Its genes in humans are called *SLC30A1* to *SLC30A10* and encode proteins ZnT1 to ZnT10, respectively (Table 1). Most of these transporters have six transmembrane domains and are located in intracellular compartments, usually associated with endosomes, Golgi complex, and endoplasmic reticulum (Huang and Tepaamorndech 2013).

The ZIP family consists of 14 transporter proteins which act to increase cell concentrations of zinc. Its genes in humans are called *SLC39A1* to *SLC39A14* and

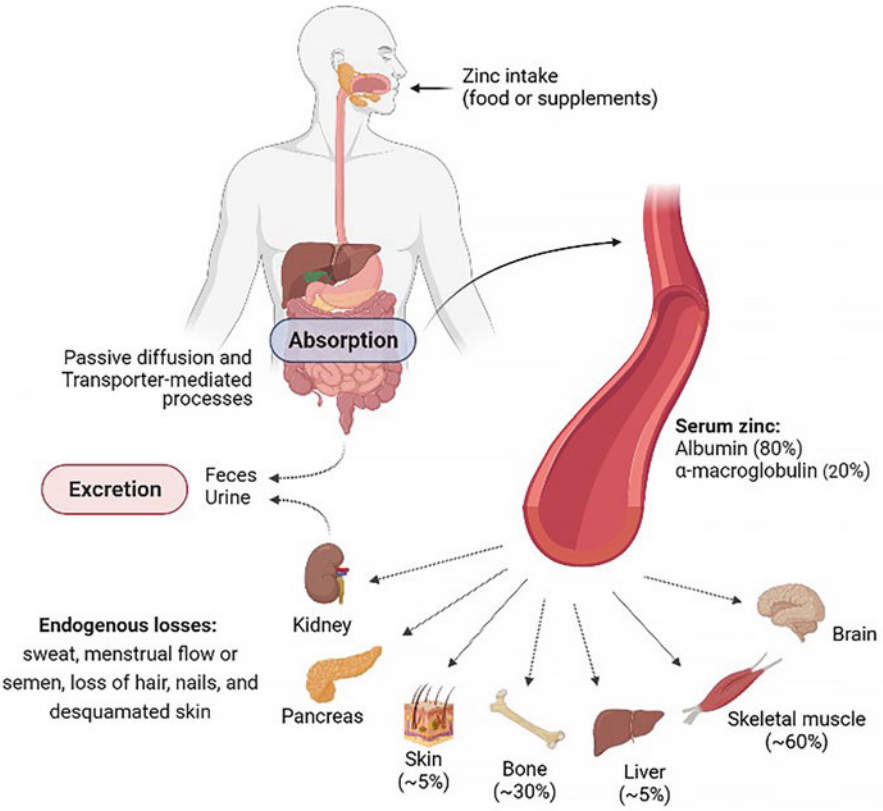

Fig. 1 Exogenous zinc absorption primarily occurs in the small intestine, which is regulated by passive diffusion mechanisms and transporter-mediated processes. After being absorbed, zinc is directed to the portal circulation, then taken up by the liver, and distributed to other tissues. Its transport in the blood is predominantly mediated by albumin and α-macroglobulin. Zinc is excreted in feces and urine, in addition to having endogenous losses (sweat, menstrual flow or semen, loss of hair, nails, and desquamated skin). Created with BioRender.com

encode proteins ZIP1 to ZIP14, respectively (Table 2). Most of these transporters have eight transmembrane domains and are located in several human intracellular compartments, including the plasma membrane, intracellular vesicles, lysosomes, the Golgi complex, and the endoplasmic reticulum (Jeong and Eide 2013).

Zinc Transporters mRNA as Biomarkers of Zinc Status

Advances in research at the molecular level have become a new tool for assessing the zinc status, enabling to evaluate the expression of transporter proteins and elucidating transcriptional mechanisms involved in zinc homeostasis. The use of the peripheral blood mononuclear cells (PBMC) transcriptome as a marker of interest in

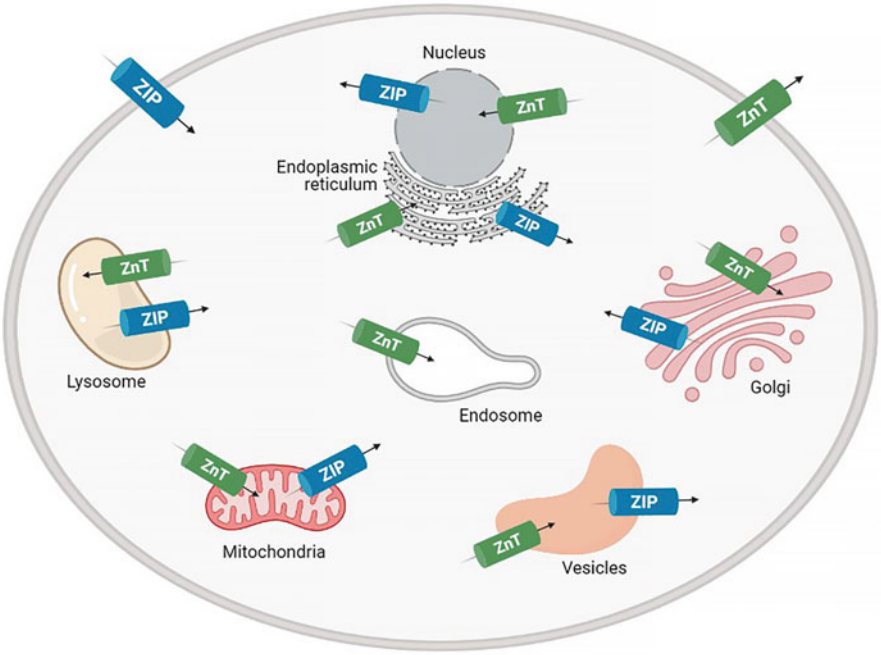

Fig. 2 Zinc transporter proteins and subcellular localization. There are two families of mammalian zinc transporters: the ZnT family (SLC30) and the ZIP family (SLC39). They transport zinc ions in opposite directions to stabilize the cytosolic zinc concentration. The ZnT family decreases intracellular zinc concentration by transporting zinc from the cytoplasm to the lumen of organelles or the extracellular space. The ZIP family acts to increase intracellular zinc concentration by transporting zinc from the extracellular space or the organelles to the cytoplasm. Created with BioRender.com

nutrigenomic studies has gained space due to its sampling and analysis ease in humans. The underlying assumption is that the transcriptomic responses of PBMC and the target tissue are similar (Reynés et al. 2018).

A significant variety of transporters present different expression patterns in response to food consumption or under different disease conditions. Thus, their understanding may help to identify subtle changes in zinc status. However, this suggestion is largely unexplored and is a promising area to investigate for potential biomarkers to assess zinc status.

Hennigar et al. (2016) performed a systematic review to compile and assess studies that determined zinc transporter and/or metallothionein expression in various blood cell types and to determine their reliability and sensitivity to change in response to zinc intake. They found that ZIP1 and ZnT1 were the most commonly measured transporters, but their changes in response to zinc supplementation or depletion were not consistent across studies.

ZnT1 is widely distributed in tissues and is more expressed in those involved with absorption, such as the small intestine, being abundant along the basolateral membrane

Table 1 SLC30A family zinc transporters: tissue/cell distribution and subcellular localization. (Adapted from Huang and Tepaamorndech 2013)

Gene	Protein name	Tissue/cell distribution	Subcellular localization
SLC30A1	ZnT1	Widespread	Plasma membrane
SLC30A2	ZnT2	Mammary gland, prostate, retina, pancreas, small intestine, kidney	Plasma membrane, endosomal/lysosomal/ secretory vesicle, mitochondria
SLC30A3	ZnT3	Brain, testes, pancreas	Synaptic vesicle
SLC30A4	ZnT4	Widespread, predominant in the mammary gland, placenta, prostate, brain, and kidney	Endosomal/secretory vesicle, plasma membrane
SLC30A5	ZnT5	Widespread, predominant in the heart, placenta, pancreas, prostate, ovary, testis, small intestine, thymus, and bone	Golgi, unknown vesicles, plasma membrane
SLC30A6	ZnT6	Widespread, predominant in the brain, lung, and intestine	Golgi, unknown vesicles
SLC30A7	ZnT7	Widespread, predominant in the intestine, stomach, prostate, retina, pancreas, testis, and muscle	Golgi, unknown vesicles
SLC30A8	ZnT8	Pancreas, thyroid, adrenal gland, testis	Secretory granule
SLC30A9	ZnT9	Widespread	Cytoplasm, nucleus
SLC30A10	ZnT10	Brain, retina, liver	Golgi apparatus

of enterocytes, where it can participate in the transfer of zinc to the bloodstream (Kambe et al. 2015). Some studies have suggested that ZnT1 mRNA is upregulated under zinc-sufficient conditions and zinc deficiency can reduce its expression (Nishito and Kambe 2019; Liuzzi et al. 2001, Langmade et al. 2000). Hennigar et al. (2021) found a high ZnT1 mRNA expression in human PBMC responsive to high or low zinc concentrations. Even so, ZnT1 mRNA showed no correlations with habitual zinc intake.

In contrast, Reis et al. (2020) found that healthy children with plasma zinc deficiency exhibited approximately 37% higher expression of ZnT1 mRNA than children with adequate plasma zinc. The authors attribute the finding to the high dietary zinc intake among these children, which was substantially above the needs, contributing to the increase of ZnT1 mRNA expression, since it is related to the reduced cellular zinc concentration, preventing cytotoxicity.

The ZIP1 transporter has the opposite function to ZnT1, being responsible for increasing intracellular zinc concentration. This transporter is widely distributed and localized in the cytoplasm membrane and intracellular vesicles. Thus, probably ZIP1 mRNA is downregulated by high zinc intake, while ZnT1 mRNA is upregulated (Kambe et al. 2021).

Andree et al. (2004) confirmed this theory reporting a decrease in ZIP1 mRNA both in cell culture of human lymphoblastoid cells and in women after administering a zinc supplement (22 mg zinc gluconate/d for 27 d). In contrast, Sharif et al. (2015)

Table 2 SLC39A family zinc transporters: tissue/cell distribution and subcellular localization. (Adapted from Jeong and Eide 2013)

Gene	Protein name	Tissue/cell distribution	Subcellular localization
SLC39A1	ZIP1	Widespread	Plasma membrane, intracellular vesicles
SLC39A2	ZIP2	Widespread	Plasma membrane
SLC39A3	ZIP3	Widespread, mammary cells, testis	Plasma membrane, lysosomes
SLC39A4	ZIP4	Gastrointestinal tract, kidney hippocampal neurons	Plasma membrane (apical surface of enterocytes), lysosomes
SLC39A5	ZIP5	Pancreas, kidney, liver, stomach, intestine	Plasma membrane (basolateral surface of enterocytes)
SLC39A6	ZIP6	Widespread	Plasma membrane
SLC39A7	ZIP7	Widespread	Endoplasmic reticulum, Golgi, intracellular vesicles
SLC39A8	ZIP8	Widespread, T-cells, erythroid, testis	Plasma membrane, lysosomes, mitochondria
SLC39A9	ZIP9	Widespread	Trans-Golgi
SLC39A10	ZIP10	Brain, liver, erythroid, kidney	Plasma membrane
SLC39A11	ZIP11	Gastrointestinal tract (stomach, cecum, and colon)	Nucleus
SLC39A12	ZIP12	Brain, lung, testis, retina	Plasma membrane, intracellular compartments
SLC39A13	ZIP13	Widespread	Intracellular vesicles, Golgi
SLC39A14	ZIP14	Widespread, liver	Plasma membrane

performed a 12-week placebo-controlled intervention trial (20 mg of zinc carnosine chelate supplement daily) and noted a significant increase in ZIP1 mRNA in the zinc-supplemented group. Other studies observed that ZIP1 mRNA did not respond to zinc supplementation or depletion in PBMC (Hennigar et al. 2021; Chu et al. 2015; Ryu et al. 2011).

These results indicate a significant variability of gene expression patterns across different populations and the design of studies about the evaluation of zinc transporters. It is important to emphasize that the evaluation of mRNA does not necessarily represent the synthesis of a particular protein, as several mechanisms are involved with the translation (Liuzzi et al. 2009). Thus, gene expression is suggestive of adaptive changes according to individual needs.

Applications to Diseases

Diabetes

The relationship between zinc and glucose homeostasis and insulin resistance (IR) is based on the insulin mimetic effect of zinc on insulin signaling. Zinc affects insulin action by modulating receptor tyrosine kinase activity and subsequent

insulin-stimulated muscle glycogen synthesis (Myers 2015). Zinc is critical for insulin storage in the secretory granules of the pancreas as an inactive zinc-insulin hexamer. A change in pH drives the dissociation of the complex into a bioactive monomer of insulin when this hexamer is released into the blood circulation (Xu et al. 2012).

Some authors have studied the role of zinc transporter expression in relation to glucose metabolism and diabetes, and the main information is summarized in Table 3. ZnT8, the product of the *SLC30A8* gene, is the zinc transporter which mediates zinc uptake into insulin and initializes zinc movement into insulin granules of the pancreatic β-cells. The expression of ZnT8 is predominantly within the pancreatic β-cells, and it is critical for the synthesis, storage, and action of insulin (Davidson et al. 2014). ZnT8 overexpression in rat insulinoma cell line (INS-1) increased glucose-stimulated insulin secretion (Chimienti et al. 2006), and down-regulation of ZnT8 in this system showed reduced insulin content and secretion in response to a hyperglycemic stimulus (Fu et al. 2009). Therefore, ZnT8 deficiency or downregulation can affect insulin biosynthesis, release, and β-cell function through direct and/or indirect mechanisms (Yi et al. 2016).

The clinical features and potential pathway of ZnT8 have been studied in the context of diabetes, including type 1 diabetes, latent autoimmune diabetes in adults (LADA), and type 2 diabetes (Yi et al. 2016). Several single-nucleotide polymorphisms (SNPs) of the *SLC30A8* gene might be dominant to environmental factors and reduce the risk of type 2 diabetes (Davidson et al. 2014.) In contrast, the C allele

Table 3 Zinc transporter expression and metabolic alterations related to glucose metabolism and diabetes. *IR* insulin resistance

Transporters	Species/model	Expression pattern	Metabolic alterations	Reference
ZnT8	Human pancreatic islet cells	↑	↑ zinc accumulation and insulin secretion in pancreatic beta-cells	Chimienti et al. (2006)
	Rat pancreatic beta-cells	↓	↓ intracellular insulin and secretion as a response to a hyperglycemic stimulus	Fu et al. (2009)
ZnT3	Rat pancreatic beta-cells (INS-1E cells)	↑	↑ pancreatic beta-cell survival ↓ insulin secretion in pancreatic beta-cell ↓ ZnT8 expression Therapeutic strategy for type 2 diabetes prevention	Smidt et al. (2016)
ZnT7	Rat pancreatic beta-cells (RINm5F)	↑	↑ insulin content and secretion in pancreatic beta-cells	Huang et al. (2010)
ZIP7	Endoplasmic reticulum	↑	↑ cytosolic zinc ↑ glucose mobilization and uptake ↓ endoplasmic reticulum stress Therapeutic strategy for IR and type 2 diabetes treatment	Adulcikas et al. (2019)

of SNP rs13266634 might remarkably downregulate ZnT8 protein expression and transporter activity, causing decreased zinc concentration and subsequently impaired β-cell function. Additionally, exocytosis of insulin granules during the biosynthesis and secretion of insulin can increase the chance of ZnT8 moving to the cell surface. This mechanism initiates ZnT8 epitope-specific T-cell-mediated β-cell destruction, triggering type 1 diabetes or type 2 diabetes and LADA (Yi et al. 2016).

Other zinc transporters, namely, ZnT3 and ZnT7, are associated with β-cell function. It has been demonstrated that ZnT3 overexpression in INS-1E cells (rat pancreatic β-cell line) resulted in decreased insulin synthesis and secretion, whereas ZnT3 overexpression improved cell survival. These data indicate opposite effects on insulin synthesis and secretion between ZnT3 and ZnT8 expression in INS-1E cells, suggesting that the upregulation of ZnT3 decreases insulin content and secretion due to the decreased ZnT8 expression in β-cells; thus, ZnT3 upregulation can be a possible therapeutic strategy in type 2 diabetes prevention (Smidt et al. 2016). By the way, the overexpression of ZnT7 in RINm5F cells (rat insulinoma cells) increased insulin content and insulin secretion, implying a positive regulation of ZnT7 on insulin synthesis and secretion (Huang et al. 2010).

The zinc transporters ZIP7 and ZIP5 have also been linked to glucose homeostasis and subsequent IR and type 2 diabetes. ZIP7 is a resident endoplasmic reticulum (ER) protein that is involved in controlling the zinc release from this organelle into the cytosol. Thus, phosphorylation and/or overexpression of ZIP7 in the ER leads to increased cytosolic zinc concentrations which activate cell signaling pathways (IRS-PI3K-AKT), stimulating glucose mobilization and uptake. Similarly, overexpression of ZIP7 inhibits ER stress which can cause IR. Therefore, strategies to target ZIP7 will have a therapeutic utility that can be beneficial in treating IR and type 2 diabetes (Adulcikas et al. 2019). Regarding ZIP5, which is located in the plasma membrane of β-cells, it was indicated that zinc transport into β-cells via ZIP5 controls insulin secretion through the increase in glucose uptake via decreased Sirtuin 1 and PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and downregulation of GLUT2 (Wang et al. 2019). These data reinforce the important role of zinc transporter expression in glucose metabolism and can be considered as therapeutic targets in the treatment of individuals with diabetes.

Cardiovascular Disease

Lower blood zinc levels have been associated with an increased risk of cardiovascular diseases (CVD) (Chu et al. 2016). Zinc exhibits anti-apoptotic, anti-inflammatory, and anti-oxidative action in vascular endothelial cells and has protective effects against endothelial damage/dysfunction. Zinc can also modulate endothelial nitric oxide synthase (eNOS) activity and NF-κB-related signaling and protect the oxidative modification of low-density lipoprotein (LDL), reducing the risk of atherosclerosis (Choi et al. 2018).

The involvement of zinc transporters expressed in vascular and cardiac cells is still unclear, as well as their biological roles in zinc homeostasis under pathophysiological conditions (Tamura 2021). The genes *SLC39A1, SLC39A7, SLC39A13, SLC39A14*, and *SLC30A9*, which encode ZIP1, ZIP7, ZIP13, ZIP14, and ZnT9, respectively, are abundant in human heart muscle tissues (Choi et al. 2018). In particular, ZIP14 is fundamental for pulmonary endothelial zinc uptake and endothelial integrity in the presence of lipopolysaccharide (LPS)-induced apoptosis (Zalewski et al. 2019). Additionally, ZIP1, ZIP13, and ZnT5 are the dominant zinc transporters expressed in human-immortalized endothelial cell lines, and smooth muscle cells contain abundant ZIP6, ZIP1, ZIP13, ZnT1, ZnT5, and ZnT9. The differences in their localization imply the characteristic roles of these zinc transporters in transferring zinc from either extracellular environment or lumen of intracellular compartments to the cell cytosol (Choi et al. 2018). Abdo et al. (2021) reported that ZIP2 and ZIP12 expression and their dysfunctions are related to regulating endothelial and vascular smooth muscle cell (VSMC) functions in response to vascular zinc deficiency and could be involved in mechanisms and management of CVD.

ZIP7 and ZnT7 are located in mitochondria beside sarco(endo)plasmic reticulum-mitochondria in cardiomyocytes and play an important role in cardiac dysfunction progression in hyperglycemic rat hearts via sarco(endo)plasmic reticulum-mitochondria uncoupling. This study highlights the contribution of altered levels of ZIP7 and ZnT7 expressions/activities to cardiac dysfunction in diabetes via affecting the subcellular labile Zn^{2+} redistribution in the hyperglycemic cardiomyocytes (Tuncay et al. 2018).

Table 4 shows the link between zinc transporters' expression pattern and some alterations in cardiovascular disease. Some evidence suggests a link between ZnT7 and atherosclerosis (Choi et al. 2018). Trimethylamine N-oxide (TMAO), the product of gut microbiome and hepatic-mediated metabolism of dietary choline and L-carnitine, is indicated as a risk factor for developing atherosclerosis (Zhu et al. 2020). A genome-wide association study identified a locus for TMAO levels on

Table 4 Zinc transporters and expression pattern related to cardiovascular diseases or alterations. *I/R* ischemia-reperfusion, *TMAO* trimethylamine N-oxide, *LDH* lactate dehydrogenase

Transporters	Disease/alteration	Expression pattern	Reference
ZnT7	Atherosclerosis related to high plasma TMAO level	Cis-expression quantitative trait locus mapped	Choi et al. (2018)
ZnT1	Atrial fibrillation	↑	Etzion et al. (2008)
ZnT1	↓ LDH	↑	Beharier et al. (2012)
ZnT1	Myocardial ischemia	↑	Bodiga et al. (2017)
ZnT2	Myocardial reperfusion	↑	
ZnT5	Myocardial reperfusion	↑	
ZnT9	Myocardial I/R	Unchanged	

chromosome 3 which co-localized with a highly significant cis-expression quantitative trait locus for ZnT7 encoding gene. This information supports that ZnT7 may be a candidate gene responsible for the association signal with atherosclerosis, but the specific role of ZnT7 in this process should be clarified (Choi et al. 2018).

The involvement of ZnT1 as a regulator in cardiac physiology and pathophysiology has been studied. A pilot study conducted with 39 patients (27 with sinus rhythm and 12 with atrial fibrillation) provide evidence of increased ZnT1 mRNA in patients with atrial fibrillation. These data indicated that ZnT1 may act as an inhibitor of L-type calcium channels during atrial electrical remodeling, suggesting ZnT1 mRNA is a contributing factor for atrial tachycardia remodeling in patients with persistent atrial fibrillation. This finding represents a relevant target for future studies in this field (Etzion et al. 2008).

Additionally, ZnT1, ZnT2, ZnT5, and ZnT9 have roles in ischemic heart diseases. ZnT1 overexpression markedly decreased lactate dehydrogenase (LDH) release and caspase activation following myocardial ischemia-reperfusion. It is speculated if ZnT1 could interact with Raf-1 kinase, which regulates the extracellular signal-regulated kinase (ERK) signaling pathway (Beharier et al. 2012). Also, the hypoxia increased the expression of ZnT1, but reoxygenation significantly increased the expression of ZnT2 and ZnT5, while ZnT9 expression remained unchanged in myocardial ischemia-reperfusion. These events suggest that ZnT9 may be necessary to maintain the normal cellular function of cardiomyocytes, while the other ZnTs are involved in the cellular response to hypoxia and reoxygenation (Bodiga et al. 2017).

The expression and role of each of the zinc transporters need to be better explored. The findings are interesting but fragmented; thus, a systematic characterization of the entire families of zinc transporters in normal, inflamed, and diseased vascular tissue is required to elucidate the relationship of zinc transporters in the context of CVDs (Zalewski et al. 2019).

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder characterized by a loss of memory due to the degeneration or loss of neurons related to cognition in the hippocampus and cortex. There is large heterogeneity in AD clinical syndromes, especially regarding the development and progression of symptoms and clinical decline. The AD pathway which leads to clinical stages of mild cognitive impairment (MCI) or dementia starts decades before the onset of symptoms (Atri 2019).

The 2011 National Institute on Aging and Alzheimer's Association (NIA-AA) criteria for AD recognizes non-amnestic AD presentations that include language, visuospatial, and executive dysfunction features (McKhann et al. 2011). Pathologically, an extracellular accumulation of amyloid plaques rich in the β-amyloid (Aβ) peptide and intracellular deposition of neurofibrillary tangles with hyperphosphorylated tau in the brain are the two major hallmarks of AD (Xu et al. 2019). Neurofibrillary tangles (NFTs) consist of intracellular (then extracellular) deposits of hyperphosphorylated tau protein, a microtubule-stabilizing protein (Atri 2019).

There is an increasing body of evidence suggesting that metal homeostasis is dysregulated in the pathology of AD. The elevated or imbalanced metal ions can induce or exacerbate Aβ overproduction, tau hyperphosphorylation, and Aβ/tau aggregation, and zinc transporters may play a critical role in AD progression (Zhang et al. 2008; Wang et al. 2020).

A pronounced difference in Zn^{2+} concentrations between brain regions is known; while subcellular compartments contain different Zn^{2+} concentrations, significant fluctuations in extracellular (synaptic) Zn^{2+} levels occur during neurotransmission. In addition, increased total Zn^{2+} levels have been detected in AD postmortem brain compared to controls. This increase correlates with advancing AD pathology (Religa et al. 2006).

The level of amyloid precursor protein (APP) and its processing pathway determine the development of AD. Zinc level modulates the function of APP and its digestion. The processing of APP relies on several activities by enzyme secretases (*α*-, *β*-, and *γ*-). The predominant route by which APP is processed in the brain is cleavage by the *α*-secretase, within the A*β* region, producing soluble amyloid precursor peptide. Also, zinc-containing transcription factors NF-κB, p53, and sp1 regulate the synthesis of APP. Notably, zinc participates in all biological activities related to the amyloid hypothesis of AD and the imbalance between production and clearance of Aβ peptides (Xie et al. 2020).

The expression of zinc transporters detected in human or mouse brains related to alterations in the progression of AD, including at least seven ZnT (ZnT1, ZnT3, ZnT4, ZnT5, ZnT6, ZnT7, and ZnT10) and six ZIP (ZIP1, ZIP3, ZIP4, ZIP6, ZIP9, and ZIP13) transporters. There are more studies on ZnT subfamily members than on ZIPs (Xu et al. 2019). Some of these studies are presented in Table 5.

Table 5 Zinc transporters and their effects on Alzheimer's disease pathology. *HPG* hippocampus/parahippocampal gyrus, *MCI* mild cognitive impairment, *EAD* early Alzheimer's disease, *LAD* late Alzheimer's disease, *AD* Alzheimer's disease, *CER* cerebellum, *PCAD* preclinical stage of Alzheimer's disease

Transporters	Localization	Clinical manifestations	Expression levels	References
ZnT1	HPG	MCI	↓	Lovell et al. (2005)
		EAD	↑	
		LAD		
ZnT4 ZnT6	HPG	EAD LAD	↑	Smith et al. (2006)
ZnT6	HPG	MCI AD	↑	Lovell et al. (2006)
ZnT1 ZnT4 ZnT6	HPG CER HPG	PCAD	↓ ↑ ↓	Lyubartseva et al. (2010)
ZnT10	Frontal cortex	AD	↓	Bosomworth et al. (2013)
ZIP1	HPG	AD	↑	Beyer et al. (2012)

A study performed with human brain specimens noted a significant decrease of ZnT1 in MCI hippocampus/parahippocampal gyrus (HPG), but a significant elevation in early-stage AD (EAD) and late-stage AD (LAD). The mechanism involved can be attributed to increased extracellular zinc, which could play a role in the precipitation of Aß and increased senile plaque deposition (Lovell et al. 2005).

ZnT4 and ZnT6 were measured in the HPG, superior and middle temporal gyrus, and cerebellum (CER) of subjects with MIC, EAD, and LAD and age-matched controls. The findings reported that ZnT4 levels located in the lysosomes and endosomes and ZnT6, which sequesters zinc to the trans-Golgi network (TGN), increased in the HPG of EAD and LAD subjects. This condition could in turn lead to the induction of Aß aggregation, driving lysosomal membrane integrity loss and possibly inducing apoptosis (Smith et al. 2006).

A preclinical stage of AD (PCAD) is described in subjects who show no overt clinical manifestations of AD but demonstrate significant AD pathology at autopsy. In this sense, a study showed ZnT1 decreased in HPG of PCAD individuals and increased ZnT4 in PCAD CER and ZnT6 in PCAD HPG, but a substantial reduction in PCAD CER compared to controls. Overall, these findings suggest that alterations in zinc transport proteins may contribute to the pathology observed in PCAD subjects before the onset of clinical symptoms (Lyubartseva et al. 2010).

In another approach, human postmortem brain tissue from Braak-staged individuals with AD displayed a reduced expression of ZnT3 mRNA (Beyer et al. 2009) and increased ZIP1 mRNA, ZnT1 mRNA, and ZnT6 mRNA in the AD cortex (Beyer et al. 2012).

The first indication that ZnT10 regulation is affected in AD was provided by Bosomworth et al. (2013), who measured ZnT10 mRNA levels in human and animal brain specimens. The authors observed a significant decrease in ZnT10 mRNA in frontal cortex samples from AD patients than from age-matched controls. Furthermore, a gender-specific effect due to the lower levels of ZnT10 mRNA was observed in female AD cases compared with controls. In parallel, they also found lower ZnT10 mRNA levels in the frontal cortex from female APP/PS1 transgenic mice. Given the subcellular localization and predicted function of ZnT10, these dysregulations may contribute to increased Aß deposition and senile plaque formation and ultimately disease progression.

There is evidence that ZIP1, ZIP3, and ZIP13 are upregulated with increasing age. Of these, ZIP1 is highly expressed in the hippocampus and murine studies, and knocking out ZIP1 attenuated seizure-induced neuronal death. The results obtained in both ZnT3 −/− mice studies demonstrated that entry of zinc into postsynaptic cells contributes to neurodegeneration in the early stages of brain injury (Qian et al. 2011). Finally, considering this overview, it is evident that altered zinc homeostasis is suggested as a risk factor for AD.

Mini-Dictionary of Terms

- **Zinc transporters.** Proteins involved in active zinc transport through cell compartments.
- **Passive diffusion mechanisms.** Cellular transport that does not involve energy expenditure and does not depend on transporter proteins.
- **Peripheral blood mononuclear cells (PBMC).** Blood cell types, basically composed by lymphocytes and monocytes.
- **Single-nucleotide polymorphism (SNP).** A genetic alteration that involves the exchange of one single nucleotide in the DNA, which may alter the translated protein.
- **Lipopolysaccharide (LPS).** Major component of Gram-negative bacteria cell wall, considered to be an endotoxin.
- **Trimethylamine N-oxide (TMAO).** The product of gut microbiome and hepatic-mediated metabolism of dietary choline and L-carnitine, indicated as a risk factor for the development of atherosclerosis.
- **Insulin mimetic.** Agents that have been shown to mimic the actions of insulin.
- **Amyloid plaques or Aβ plaques.** Extracellular deposits of β-amyloid (Aβ) peptides which may play a critical role in the progression of AD when there is exacerbated overproduction.
- **Neurofibrillary tangles (NFTs).** Insoluble twisted fibers found inside the brain's cells which primarily consist of accumulations of hyperphosphorylated tau protein.

Key Facts of Zinc Transporters in Diabetes

- Zinc transporters' expression can affect zinc accumulation and insulin secretion in pancreatic beta-cells.
- Zinc transporters can be considered as therapeutic targets for treating individuals with diabetes.
- ZnT8 protein expression and transporter activity are associated with the risk of diabetes.
- ZnT3 overexpression can decrease ZnT8 expression in β-cells.
- ZIP7 overexpression is related to preventing insulin resistance.

Key Facts of Zinc Transporters in Cardiovascular Disease

- Zinc transporters are abundant in human heart muscle tissues and could be considered in managing cardiovascular disease.
- ZnT7 may be a candidate gene related to atherosclerosis.

- Individuals with persistent atrial fibrillation have increased ZnT1 expression, and this transporter can be a relevant target for future studies in this field.
- ZnT1, ZnT2, ZnT5, and ZnT9 expression are involved in mechanisms related to ischemic heart disease.
- The expression and role of each zinc transporter need to be better explored considering the characterization of entire families of the zinc transporters in normal, inflamed, and diseased vascular tissue.

Key Facts of Zinc Transporters in Alzheimer's Disease

- Increased total Zn^{2+} levels have been detected in AD postmortem brain compared to controls, with the increase correlating with advancing AD pathology.
- At least seven ZnT and six ZIP transporters in the brain are related to alterations in AD progression.
- The main question is explaining why the expression levels of Zn transporters alter at different AD stages.
- Individuals with AD seem to have a reduced expression of ZnT3 mRNA and increased ZIP1 mRNA, ZnT1 mRNA, and ZnT6 mRNA in the AD cortex.
- A significant role of zinc transporters in AD pathology and their multiple pathological changes in disease progression questions remain to be resolved.

Summary Points

- Zinc transporters are divided into two families: the ZnT family (SLC30) and the ZIP family (SLC39).
- The ZnT family consists of ten transporter proteins that act to decrease intracellular zinc levels.
- The ZIP family consists of 14 transporter proteins that act to increase cell concentrations of zinc.
- The zinc transporters present different expression patterns in response to zinc intake or under different nutritional statuses.
- Different diseases such as diabetes, cardiovascular disease, and Alzheimer's disease affect the expression of several transporters, being correlated with their diagnosis or prognosis.

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24 Circulating MicroRNA (miRNA)s as Biological Markers and Links with Obesity and Obesity-Related Morbid Conditions

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Abstract

In late years, a substantial advance has been made in the study of the role of microRNAs (miRNAs) in the pathogenesis of diseases. New evidence shows that dysregulation of miRNAs represents an etiologic factor of a variety of disorders, including cancer. Besides, miRNAs have also emerged as fundamental regulators of metabolic processes taking part in maintaining energy balance and metabolic homeostasis. Dysregulation of miRNAs, by directly affecting the status and functions of adipose tissue, pancreas, liver, and muscle, contributes to metabolic abnormalities being fully implicated in body fat accumulation, obesity, and obesity-related diseases. The discovery of circulating miRNAs has highlighted their potential as endocrine signaling molecules and disease indicators. Nevertheless, the study of the involvement of miRNAs in metabolic dysfunctions is still a young field of research and information on their role is nearly limited to date. As well, the contemporary rising in childhood obesity rates creates a need for tools that quantify metabolic changes in obese children and adolescents for the early detection or prevention of comorbidities. This chapter aims to provide current insights into the role of miRNAs as biological markers focusing on their link to obesity and obesity-related morbid conditions.

Keywords

Disease biomarkers · MicroRNA · miRNA · Obesity · Metabolic disease · Metabolic syndrome · T2DM · Non-communicable diseases · Energy balance · Metabolic homeostasis

Abbreviations

AGO	Argonaute
BMI	Body Mass Index
BMPs	bone morphogenetic proteins
C/EBPs	CCAAT/enhancer-binding proteins
cmiRNAs	circulating miRNAs
DGCR8	DiGeorge Syndrome Critical Region 8
GWA	genome-wide association (studies)
HbA1c	glycated hemoglobin A1c
HNF	hepatocyte nuclear factor
HOMA-IR score	homeostatic model assessment for insulin resistance
LETFs	liver-enriched transcription factors
miRNAs	microRNAs
ncRNAs	non-coding RNAs
NGS	next-generation sequencing
NPM-1	nucleophosmin-1
PPAR-γ	proliferator-activated receptor-γ
RISCs	RNA-induced silencing complexes
SFA	saturated fatty acids

SREBP1	sterol regulatory element-binding protein
T1DM	type 1 diabetes mellitus
T2DM	type-2 diabetes mellitus
TGF-β	transforming growth factor beta
Wnt	Wingless/Integrated

Numerous published studies examined different aspects of the complex etiology of obesity with reference to miRNAs deregulation. Utilizing scientific evidence from these studies, this chapter aims at carrying an in-depth analysis of candidate miRNAs as biological markers of obesity and obesity-related morbid conditions.

Introduction

The obesity epidemic represents a key health challenge worldwide since it is connected with severe concerns for human health. This multifaceted condition affects virtually all age and socioeconomic groups in low-income and middle-income countries. Its prevalence has steadily increased over the last 30 years, most likely due to adverse changes in environmental and demographic factors.

According to the World Health Organization WHO, there are about two billion overweight adults in the world, and more than 600 million of them are considered obese. In the USA, over 60% of adult Americans are overweight or obese, and most countries around the world have experienced similar growth rates of obesity in recent times. In general, men have higher rates of overweight, whereas women are more usual to have higher taxes of obesity. In this context, meta-analysis studies showed that children with high BMI are more likely to have obesity in adulthood compared to normal-weight children (Simmonds et al. 2015).

Epidemiological studies have established the firm association between increased BMI and quality/expectancy of life, diabetes, hyperlipidemia, hypertension, heart disease, stroke, non-alcoholic fatty liver disease, cancer, psychological consequences, and other pathologic conditions. Dramatically, up to four million deaths worldwide can be linked to overweight and obesity, and more than two-thirds of these deaths are related to cardiovascular events. Yet, type 2 diabetes (T2DM) denotes a major health concern since its prevalence has rapidly increased for the last, accounting for 90–95% of total diabetes worldwide, both in adults and children. Not all people with T2DM are overweight or obese, but the majority are. While most subjects who suffer from obesity are not diabetic, the occurrence of T2DM increases as adiposity rises, hence, the term "diabesity" has been coined in recent times.

The augmented adiposity stimulates the production of pro-inflammatory cytokines which leads to a chronic inflammatory status that finally results in insulin resistance and other metabolic changes. Weight control remains the key to the prevention and management of this condition.

The obesity epidemic is considered a critical public health issue worldwide, its subsequent concerns are not restricted to industrialized societies, paradoxically

coexisting with undernutrition, even though a plateau in prevalence rates has been reported for childhood obesity in several Western countries. Besides, the secular changes in energy intake and expenditure invoked as the primary cause of obesity, several loci on the human genome have also been associated with obesity predisposition. Studies in twins have advised that genetic background may explain about 40–80% of the variation in obesity susceptibility. Interestingly, most loci linked to obesity predisposition likewise are associated with food intake and energy balance control (Locke et al. 2015).

Despite extensive in-depth GWA studies on obesity genetics, most of the genetic variability in BMI remains unexplained and the influence of single candidate genes is, to a certain extent, restricted. Further investigations have proposed that obesity-predisposing genes are not deterministic, but they interact with a multiplicity of individual and lifestyle factors, including obesogenic environments, low education levels, sedentary lifestyle, sleep, and others (Huls et al. 2021). When considering environmental factors, crucial factors contributing to the epidemics are considered either the drastic changes in the quantity and quality of food included in the diet (energy density) or the decline in the level of energy expenditure in terms of physical activity. Comprehensively, obesity is sustained by three major mainstays involving the environment and the behavior in addition to multiple gene variants additively conferring a different degree of susceptibility (Stranger et al. 2011). However, environmental factors are likely to be the driving factors in the obesity epidemic.

An Overview of White Adipose Tissue

The basic tissue for energy storage in humans is the white adipose tissue. Aside from its storage function, this tissue is metabolically active, releasing hundreds of different factors such as hormones like leptin and adiponectin, growth factors like IGF-1 and PDGF, and inflammatory mediators like IL-6, IL-8, or TNF-α, all of which playing a role in insulin sensitivity and appetite regulation.

Chronic low-grade inflammation is a key characteristic of obesity that leads to insulin resistance in target organs such adipose tissue, liver, muscle, and the vascular system. Excessive calorie intake mainly derived from carbohydrates and fats can prompt inflammatory reactions that can negatively affect metabolic functions while also increasing stress and inflammation. As a result, the nutrition-immunity theory proposes that obesity caused by nutrient excess stimulates low-level inflammatory processes in adipose tissue. The condition is linked to an increased macrophage enrolment and immune cell proliferation/activation/infiltration as well as to adipocyte hypertrophy and impaired adipogenesis.

Adipogenesis is a complex process involving cell commitment, clonal growth, and terminal differentiation to develop fibroblast-like pre-adipocytes into mature adipocytes. A variety of regulatory mechanisms, including extracellular circulating hormones, endocellular transcription factors, and post-transcriptional modulators of gene expression govern this process. The transition from pre-adipocytes to mature adipocytes requires some transcription factors, comprising the peroxisome

proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs), which control the gene expression leading to adipocyte phenotypes. C/EBPβ and C/EBPδ are the primary adipogenesis regulators and are triggered by adipogenic stimuli. These factors target promoters of genes encoding critical adipogenic factors such as C/EBPα, PPARγ, in addition to SREBP1 (sterol regulatory element-binding protein) which is the main lipogenic gene regulator. C/EBPα interacts with specific promoters and enhancers as a homodimer or by generating heterodimers with C/EBPβ and C/EBPγ factors. Besides, C/EBPα is required for pre-adipocyte development into mature adipocytes. This factor, among the various targets, by interacting with the promoter of the leptin gene, modulates its expression playing a substantial role in body weight homeostasis.

PPARγ directly activates C/EBPα transcription and, on the other hand, C/EBPα stimulates PPARγ transcription in a virtuous loop promoting adipogenesis. Together, PPARγ and C/EBPα help in assisting the expression of genes involved in insulin sensitivity, lipogenesis, and lipolysis, supporting the mature adipocyte terminal differentiation. The anti-adipogenic signaling cascade, controlled by BMPs, TGF-β, Wnt, and hedgehog, is correspondingly part of the dynamic set of events controlling the cell commitment and adipocyte development. Noticeably, an increase in adipocyte volume (hypertrophy) and number (hyperplasia) leads to higher fat mass and energy storage levels in adipose tissue, contributing to an increased risk of obesity.

miRNAs: Small RNAs Playing a Central Role in Gene Expression Control

Histone modifications and chromatin remodeling mechanisms are controlled by a variety of dynamic systems that assist the complex network of the epigenetic control of gene activity. There have been found hundreds of post-translational modifications of core histones, including acetylation, phosphorylation, methylation, and ubiquitylation. In essence, any step in the gene expression flow is wisely regulated, and the discovery of small non-coding RNAs (sncRNAs) has introduced new contributors to the well-diversified set of supervisory mechanisms. Epigenetics is typically described as a heritable change that occurs in the DNA without affecting its sequence. Recent improvements in epigenetic analysis techniques have made a significant contribution to the field's progress. Nonetheless, research into the role of epigenetics in obesity is still in its youth.

In this context, numerous studies suggest a striking interconnection between miRNAs deregulation and diseases. miRNAs have emerged as body homeostasis peacekeepers, playing critical roles in the physiopathology of a variety of processes, including body energy balance and metabolic homeostasis. New studies have recognized that miRNAs are causally associated with obesity, metabolic syndrome, T2DM, and other non-communicable diseases (Condrat et al. 2020).

The sncRNAs are categorized as small interfering RNAs, PIWI-interacting RNAs, endogenous small interfering RNAs, promoter-associated RNAs, small nucleolar RNAs, and microRNAs (miRNAs) based on their working mechanism,

assembly, and structure. miRNAs are sncRNAs (20–24 nucleotides in length) acting as post-transcriptional regulators of gene expression by base-pairing with target mRNAs. Each miRNA can bind to several mRNAs, and a single transcript may have many miRNA recognition sites. Up to 2599 different human miRNAs have been deposited in miRTarBase (Huang et al. 2020). The release 22.1 of the repository includes 38,589 entries representing hairpin precursor miRNAs that express 48,885 mature miRNA in 271 species.

In just a few years, knowledge of miRNA has proceeded from a single paper reporting the discovery of a noncoding RNA in *C. elegans* (Lee et al. 1993) to thousands of papers demonstrating their crucial role in a wide range of disorders and physiological processes. miRNAs are relevant components of the cellular epigenetic machinery that act as specific gene silencers by base-pairing to the 3′ untranslated sequence of a target mRNA, but they have also been shown to bind anywhere along the target mRNA sequence. miRNAs work by either suppressing translation or affecting the stability and degradation of the mRNA. The nucleotides in positions 2–8 of a miRNA have been referred to as "*seed sequence*" because they are required for base pairing with a target mRNA. New developments in a transcriptome-wide method of mapping miRNA binding sites revealed that non-canonical seed-like patterns also mediate a high proportion of miRNA-target in in vivo interactions.

The similarity in seed region has also been used to group miRNAs into "families" with the ability to target common clusters of mRNA transcripts. Although certain miRNAs have tissue-specific localization, the majority of miRNAs exhibit a broad tissue distribution.

Remarkably, a single miRNA can regulate groups of transcripts at the same time, and a single mRNA typically includes numerous interaction sites for many miRNAs, so establishing intricate regulatory circuits. Although a single miRNA has a limited modulatory effect on a particular target, its activity frequently influences several transcripts in a signaling network consequently exercising powerful cumulative effects: in this perspective Metazoan miRNAs have been designated as the "sculptors" of the transcriptome (Bartel 2018).

Endogenous miRNAs are capable to alter the expression of up to 60% of mouse and human genes, according to scientific evidence. As a result, miRNAs have been connected to a wide range of conditions, both in healthy and diseased states. Recently, a collection of knockdown phenotypes representative of critical activities of miRNAs has been reported (Bartel 2018). Finally, new evidence point to the concept that dysregulation of miRNAs represent an etiologic factor and/or indication of a variety of disorders, including cancer.

miRNAs can also be packaged and secreted from cells arranged either into protein or exosomes complexes. The identification of about 300 circulating miRNAs in plasma and other bodily fluids has emphasized their potential as relevant intercellular signaling molecules and disease indicators (Witwer 2015). As a result, dysregulation of miRNAs has been established to reflect the condition and functions of various tissues and organs, probably contributing to their abnormalities (Iacomino and Siani 2017). Their diagnosis and prognostic potential of miRNAs as non-invasive biomarkers has been advised, either as individual biomarkers or in combination.

Biogenesis of miRNAs and Mechanisms of Action

The biogenesis of miRNAs has been widely characterized in recent times (Fig. 1) (Finnegan and Pasquinelli 2013). Transcription of miRNAs, as well as protein-coding genes, is finely controlled by chromatin status and transcription factors. miRNAs coding genes can be found in both introns and exons of protein-coding genes (approximately 40% of mammalian miRNAs are found within introns of protein-coding genes) as well as in intergenic regions, and are evolutionary conserved.

Pri-miRNAs are transcribed, together with the host gene (intragenic miRNAs) using the genomic DNA as the template by the RNA polymerase II, or independently of the host gene with the use of own promoters (intergenic miRNAs), as large primary transcripts long several hundred base pairs. Multiple loci code for miRNAs some of which are organized in co-transcribed clusters. The pri-miRNA displays a typical structure characterized by a hairpin and three spiral turns, flanked by a single-stranded RNA. The enzymatic complex, including the RNA-binding cofactor DiGeorge Syndrome Critical Region 8 (DGCR8, Pasha) and the RNase III Drosha enzyme (a double-strand endonuclease), can recognize this configuration. The deriving product, a 60–70 nucleotide long precursor (pre-miRNA), is exported to the cytoplasm by interacting with Exportin-5 and Ran GTPase, where it is processed

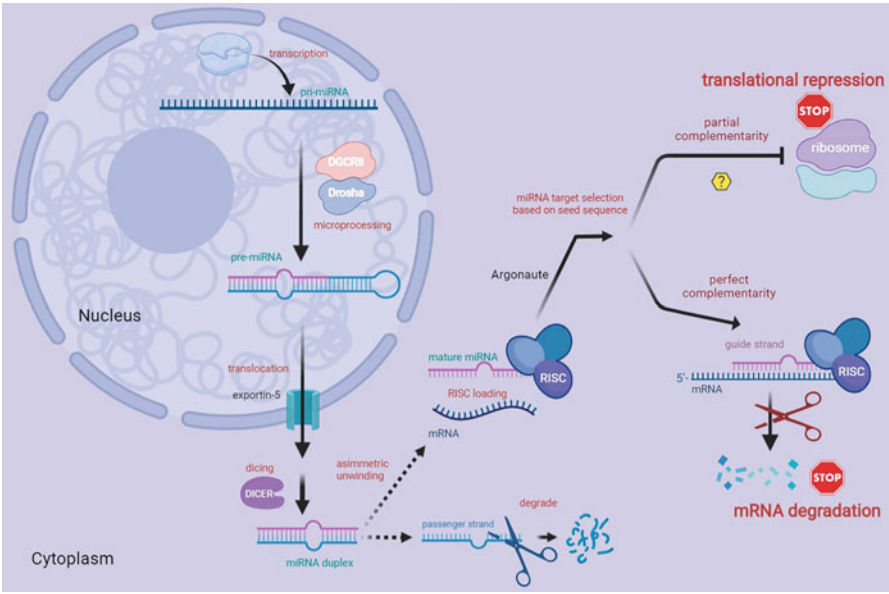

Fig. 1 miRNAs are transcribed from gDNA by RNA polymerase II (pri-miRNA). The complex Drosha-DGCR8 processes the pri-miRNA to pre-miRNA. Exportin-5 translocates the pre-miRNA to the cytoplasm where it is processed to a miRNA duplex by the DICER complex. The duplex unwinds and the mature miRNA assembles into the RISC. The miRNA guide strand targets mRNA by base-pairing so determining gene silencing via mRNA cleavage or translation repression on the base of identity between the miRNA "seed" to the 3′ UTR target sequence of the mRNA

by the enzyme Dicer RNase III, which further cleaves the pre-miRNA into a 19–24 nucleotide long mature miRNA duplex. The duplex is unwound and only one strand produces the mature miRNA sequence. This last is then integrated into the RISCs (RNA-induced silencing complexes) ribonuclear complexes which incorporate the Argonaute (AGO) family proteins that identify target mRNAs by complementary base pairing, resulting in post-transcription gene silencing either by translation suppression or destruction of the target transcript. Ago2 activity cuts the target RNA between nucleotides corresponding to positions 10 and 11 of the guide strand when the miRNA and target mRNA are fully complementary. Else, the RISC promotes a flow of inhibitory processes leading the targets to canonical degradation pathways.

Interestingly, both spliced and unspliced transcripts may work as primary miRNA transcripts for some exonic miRNAs, such as miR-22, miR-155, and miR-146a. Of note, the interactions between specific miRNAs and their mRNA targets can be disrupted by genetic and somatic changes in miRNA motifs. Mutations, on the other hand, can enable the miRNA to interact with a new range of targets, redirecting the miRNA regulatory networks.

miRNAs Detection

miRNAs profiling is a fundamental step that requires sensitive, specific, and reproducible detection systems. Different methods, such as Next-Generation Sequencing (NGS), microarray, reverse transcription-quantitative PCR, and others (Cai et al. 2021), have been settled to quantify miRNAs in biological samples, with each method having its benefits and disadvantages (Mestdagh et al. 2014). However, their profiling is puzzling because their small size, sequence similarities, and low abundance. Of note, miRNAs embrace about 0.01% of total RNA (Dong et al. 2013), and single miRNA is present from a few to tens of thousands of copies per cell (Bartel 2004). Furthermore, miRNAs can differ by a single nucleotide, also taking into account variations between mature miRNAs and their precursors, besides, each miRNA can be differentially expressed in the course of disparate cellular processes or disease conditions.

Accordingly, miRNA recognition approaches must be highly specific and capable to identify very limited target molecules among an abundance of strictly related RNA molecules. Circulating miRNAs detection might be difficult due to unwanted contaminant inhibitors that can interfere with downstream quantification techniques as hemoglobin in blood-derivative samples or cellular components that can potentially introduce a detection bias.

Therefore, inconsistencies found between different studies in the field could be, at least in part, explained by differences in extraction and identification procedures, experimental setup, and data processing, pointing to the importance of developing well-standardized protocols and operative guidelines.

miRNAs Target Prediction

With millions of hypothetically conceivable interactions among miRNAs and mRNAs, the known human miRNAs targetome is far to be determined (Kern et al. 2021). Identification of miRNA interaction with a target mRNA remains a concern because of multiple target recognition. Bioinformatics tools, geared at a subsequent bench validation of expected mRNA interactors, are frequently used to get over this problem. Computational approaches predicting miRNA targets often identify binding sites that have been retained evolutionarily. Current methods, on the other hand, frequently reveal challenges to achieve effective performance due to high false-positive and false-negative detection rates. With these limits, target identification remains an essential and stimulating step preceding functional annotation analysis (Kern et al. 2020).

The Web-page (http://mirnatools.eu/index.html) includes a number of useful links for miRNAs interaction analysis.

The Role of miRNAs Across Tissues in the Context of Obesity

Numerous researches have linked dysregulation in miRNA profiles in tissues (e.g., pancreas, adipose tissue, and liver) to body fat accumulation, obesity (Fig. 2), and a multiplicity of metabolic alterations. miRNAs play a crucial role in maintaining energy

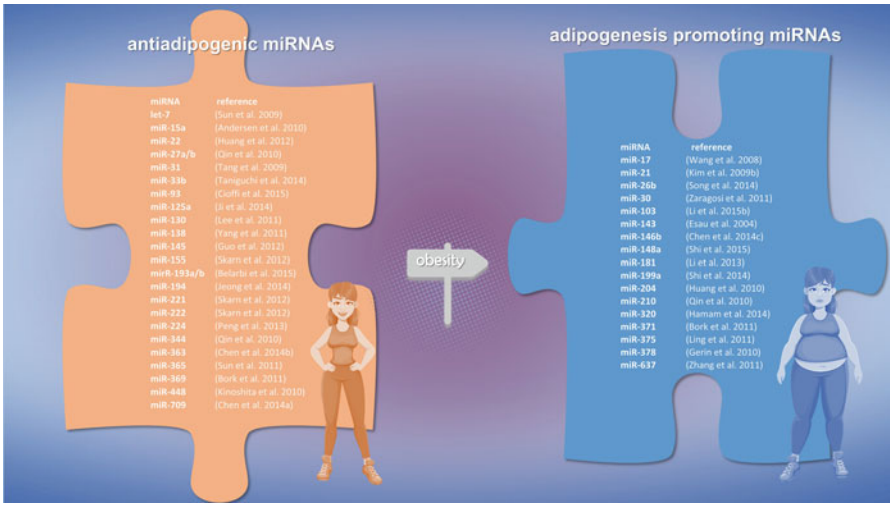

Fig. 2 miRNAs' contribution to adipose tissue development and physiopathology. A restricted example is reported with some miRNAs interfering with adipocytes differentiation and others promoting adipogenesis

balance and metabolic equilibrium in living organisms by regulating different metabolic pathways. Abnormal fat storage upturns a condition of subclinical chronic inflammation, therefore, increasing pathogenic risks. This scenario is connected to an enhanced macrophage recruiting and immune cell proliferation-activation-infiltration as well as to adipocyte hypertrophy and altered adipogenesis. Endo-cellular transcription factors, extracellular circulating hormones, and further post-transcription regulators are among the supervisory stimuli that keep this last mechanism into account.

A study of miR-14 function in Drosophila first found that miRNAs negatively affect fat metabolism by targeting p38 and MAPK (Xu et al. 2003). This was the earliest evidence of the role of miRNAs in influencing fat cells. Then, a variety of miRNAs functions in influencing glucose, lipid metabolism, adipocyte differentiation, as well regulating cell mass and the insulin-signaling pathway was established both in physiological and pathological conditions.

Many factors play roles in controlling the different phases of adipogenesis, and new research suggests that miRNAs have a relevant part in adipose tissue formation and its physiopathology. As a simplified example, Fig. 2 shows that specific miRNAs inhibit adipocyte development, whereas others enhance adipogenesis (Price and Fernandez-Hernando 2016; Iacomino et al. 2020). However, there is uncertain evidence around the accurate processes by which miRNAs act in this context.

Recently, Thomou et al. discovered a further implication of adipose tissue in the cell crosstalk process (Thomou et al. 2017). Researchers found that adipose tissue acts as a key source of circulating miRNAs, which operate as metabolic regulators and affect gene expression in distant organs. Besides, Li et al. found that extracellular vesicles released by adipose tissue contained miRNAs acting in intercellular signaling. In detail, miR-221-3p enables a mechanism by which perivascular adipose tissue stimulates the early-stage vascular remodeling in the context of obesity-related inflammation (Li et al. 2019).

Several miRNAs direct pancreatic development and have been reported elsewhere (Kredo-Russo et al. 2012). Insulin and glucagon release by the endocrine pancreas have a critical part in maintaining glucose homeostasis. Diabetes is causally linked to changes in the production and function of these hormones. T2DM is a multifaceted condition characterized by pancreatic islet dysfunction and insulin resistance in peripheral tissues. In T2DM, a decrease in β-cell function/mass has been associated with lower insulin levels. Dedifferentiation and identity of β-cell also contribute to insulin production reduction. In pancreatic islets, miR-375 is the most abundant miRNA, and its presence is required for successful tissue differentiation (Lu et al. 2021). During the maturation of pancreatic islet cells, miR-375 is widely expressed, and its lowering is interconnected to the activity of β-cells. During pancreatic development, miR-375 targets a number of genes involved in the cell growth functioning of islets (Avnit-Sagi et al. 2009). Inhibition of miR-375 induces improved insulin secretion, while miR-375 overexpression attenuates insulin release by targeting myotrophin, a protein involved in insulin granule fusion, by inhibiting exocytosis (Poy et al. 2004). Concomitantly, it has been reported that miR-375 reduced insulin levels by targeting the phosphoinositide-dependent kinase-1 (El Ouaamari et al. 2008). Additional research also found that pancreatic β-cells

are involved in exporting miR-375 to HDL and that this process is inversely connected to insulin secretion (Sedgeman et al. 2019). Higher expression of blood miR-375 was detected in T2DM patients (Garcia-Jacobo et al. 2019). Remarkable, islet-specific miRNAs are a hot topic for detecting islet stress or degeneration. It has been discovered that throughout the early stages of prediabetes, stressed islets release miRNAs with consistent signatures that are distinctive to each stage (Vasu et al. 2019). In this context, miRNAs have been recognized as driving epigenetic elements contributing to glucose homeostasis interconnected to both T1DM and T2DM conditions.

Skeletal muscle accounts for over 40% of total body mass, and it is the greatest consumer of glucose, accounting for nearly 75% of insulin-mediated glucose adsorption. Because of the beneficial effects on insulin resistance and T2DM, regular physical exercise and fitness contribute to successful improvements in health status. The myomiR family of miRNAs has a high level of expression in skeletal muscle functioning as modulators of skeletal and cardiac myogenesis, and are associated with muscle proliferation, metabolism, exercise, and hypertrophy. MyomiRs are transcriptionally controlled by myogenic regulatory factors and comprise miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499 (McCarthy 2011). Of note, miR-206 is preferentially expressed in skeletal muscle while miR-208a is primarily found in the heart. Besides, most of these miRNAs are co-expressed in both heart and skeletal muscles. miR-208a also regulates glucose metabolism and energy homeostasis. Attractively, oligonucleotides anti-miR-208 confer resistance to diet-induced obesity and improve glucose tolerance in mice (Grueter et al. 2012).

miRNAs also govern a variety of liver activities and evidence indicates that they as well play a role in liver diseases (Szabo and Bala 2013). With roughly 135,000 copies, miR-122 is the dominant hepatocyte-specific miRNA that accounts for about 75% of overall miRNAs in hepatocytes, making it one of the most highly expressed in humans. Liver-enriched transcription factors (LETFs), such as hepatocyte nuclear factor (HNF) 6 and 4a, regulate miR-122 levels. This miRNA regulatory network has been implicated in a variety of activities in the liver, including cholesterol metabolism, stress responses, viral infections, cancer, and circadian hepatic gene regulation (Tsai et al. 2012). In both humans and animal models, miR-122 has shown a starring role in metabolic syndrome and other liver diseases such as alcohol-related liver inflammation, autoimmune processes, and the development of liver fibrosis. Besides, hepatocellular carcinoma, nonalcoholic steatohepatitis, and liver cirrhosis have all been linked to the pathological suppression of miR-122.

Given the differential effects and multifaceted expression of miRNA in specific tissue/organs in the context of obesity, more details on this topic can be found elsewhere (Iacomino and Siani 2017; Iacomino et al. 2020) and a partial list of tissue-relevant miRNAs is summarized in Fig. 3.

Contemporary researches have further supported the idea that nutrition and lifestyle variables also alter miRNAs expression (Slattery et al. 2017), and numerous microRNA families have been linked to a variety of dietary treatments. Accordingly, NutrimiRomics is a new discipline, inspired by the impact of the diet on miRNAs levels and their sub-sequential effects on gene expression and health status.

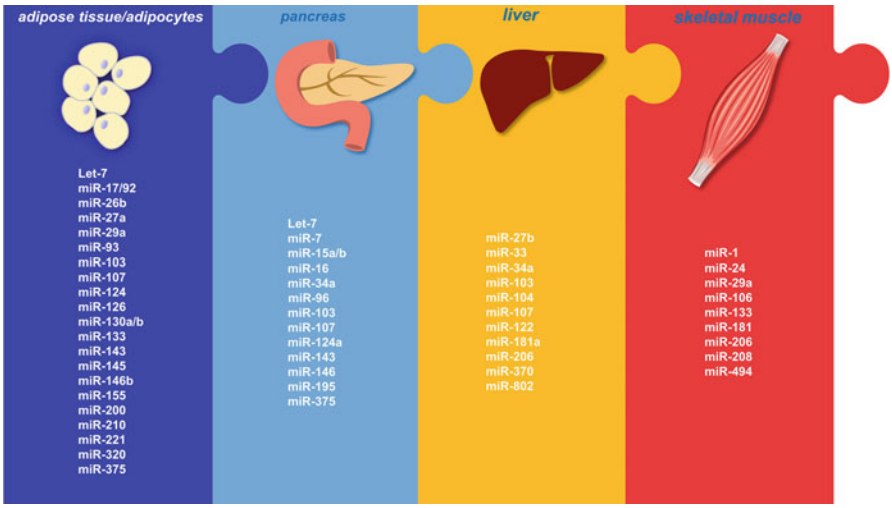

Fig. 3 Changes in miRNA profiles in different tissues are linked to obesity and metabolic diseases (a partial list)

Besides, dietary miRNAs are predicted to survive digestion through the gastrointestinal tract (Liang et al. 2014). Nevertheless, the role and ability of food-related miRNAs to interfere with cross-species mRNA remains enigmatic.

Circulating miRNAs in Obesity and Obesity-Related Comorbidities

miRNAs were first discovered in cells and an increasing number of miRNAs have lately been discovered in unusually high concentrations in blood and other bodily fluids such as maternal milk, serum, saliva, plasma, and urine. Given the ubiquity of nucleases, the hypothesis that miRNAs remained unexpectedly stable in blood and bodily fluids was met with skepticism by the scientific community. Nonetheless, the discovery prompted interest in the possibility of using changes in cell-free miRNAs as non-invasive biomarkers for a number of disorders. Whole blood, serum, and plasma are the most common sources of miRNAs, and an increasing number of studies suggests that these molecules can be employed as early diagnostic tools to resemble physiological states as well a variety of diseases and metabolic conditions.

Circulating miRNAs (cmiRNAs) are not naked molecules, as one might expect, and two key strategies have been found to protect them from nuclease activity. The first involves the formation of complexes of specific proteins, such as Argonaute 2 (AGO-2) (participating in the RNA silencing complex), high-density lipoproteins, or nucleophosmin-1 (NPM-1) (an RNA-binding protein taking part in ribosome nuclear export). The discovery of cmiRNAs inside circulating microvesicles or exosomes, deriving either from the cell plasma membrane or from endosomal compartments, represents the additional reported mechanism. Even though a

molecular basis for miRNA release from donor cells is still unknown, increasing evidence suggests that extracellular miRNAs, structured in exosomes or protein complexes, can be transported to recipient cells and influence the translation of the target gene/s. Despite this, little is known about the functional significance of cmiRNAs, with many studies suggesting that they can play a role in tissue communication.

Distinct cmiRNA profiles have been reported in patients with obesity and other metabolic diseases, and levels of certain cmiRNAs change depending on the context.

For instance, one of the first investigations found that circulating miR-126 is reduced in T2DM (Zampetaki et al. 2010). Further studies found elevated serum levels of miRNA- 192 and 194 in incident T2DM (Jaeger et al. 2018), or that after myocardial infarction miR-1, miR-21, miR-133a, and miR-208 were enriched in plasma (Zile et al. 2011), as well as miR-122 raised in hepatic damage and steatosis (Cermelli et al. 2011), in addition to let-7e in hypertension (Li et al. 2011).

miR-29a, miR-34a, miR-103, miR-107, miR-132, miR-142-3p, miR-144, and miR-375 are promising circulatory biomarkers for T2DM, according to a meta-analysis by Zhu and Leung (2015). miR-199a-3p and miR-223 were discovered as possible T2DM tissue biomarkers in the same study. In people with T2DM, levels of circulating miR-27a, miR-29a, miR-142-3p, miR-222, miR-320a, and miR-375 increased, while levels of miR-17, miR-20b, miR-197, and miR-652 decreased, according to another meta-analysis (Villard et al. 2015).

Platelet activity has been reported to be higher in diabetic patients. Given the abundance of platelets in the blood and their large contribution to the cmiRNA pool, these findings encouraged the idea that platelet-derived miRNAs could be used as biomarkers in inflammatory disorders such as T2DM and CVD (Pordzik et al. 2018). Although the relative levels of miRNA in platelets between T2DM and control subjects were not substantially different in a study (Stratz et al. 2014), the functional relevance of these molecules remains crucial in the etiology of T2DM (Pordzik et al. 2019).

It was recently reported that metformin treatment affected several miRNAs encapsulated in circulating extracellular vesicles in T2DM patients, suggesting that these miRNAs could be used as biomarkers to measure drug treatment responsiveness (Ghai et al. 2019).

Furthermore, in the high-risk Asian Indian ethnic group, miR-191 was positively associated with glycemic impairment and progression; so far, miR-122, miR-15a, miR-197, miR-320a, miR-423, and miR-486 had an inverse relationship with the odds of glycemic progression after 2.5 years compared to stable group (Flowers et al. 2015).

Levels of miR-144-5p, miR-122-5p, miR-148a-3p, miR-589-5p, and let-7a-5p were found to be linked to glycemic status in a recent population-based study (Mononen et al. 2019). miR-144-5p and miR-148a-3p were linked to glucose levels in the same study, whereas miR-144-5p, miR-122-5p, miR-184, and miR-339-3p were linked to insulin levels and the HOMA-IR score, as well miR-148a-3p, miR-15b-3p, miR-93-3p, miR-146b-5p, miR-221-3p, miR-18a-3p, miR-642a-5 were associated to HbA1c levels.

An additional investigation found that cmiRNA levels combined with HbA1c are useful in predicting T2DM development in adults (Jimenez-Lucena et al. 2018). Besides, miR-126 has been reported to be a valuable marker for metabolic diseases in children (Krause et al. 2015). A number of investigation has described that other cmiRNAs are linked with T2DM including miR-15a (Jimenez-Lucena et al. 2018), miR-126 (Zhang et al. 2013), miR-146a (Balasubramanyam et al. 2011), miR-375 (Kong et al. 2011), among the others. miR-298, miR-491-5p, and miR-1307-3p have also been recognized, as predictive of T2DM in prediabetes subjects (Sidorkiewicz et al. 2020; Nigi et al. 2018). Parrizas et al. described specific cmiRNA profiles for prediabetes by identifying two different miRNAs enclosed inside microvesicles, miR-10b, and miR-223-3p, whose combination was able to distinguish between progressor and non-progressor prediabetic subjects at a point when other biomarkers, such as glycemia, were not beneficial in distinguishing them (Parrizas et al. 2020). This study emphasized the value of miRNA screening as a tool for those at risk of developing diabetes.

Similarly, in the recent past, our research group found that plasma miR-191-3p and miR-375 are related to early variations in glycemic homeostasis in healthy overweight/obese children and adolescents (Iacomino et al. 2021). Of note, no one of the study-subject suffered from metabolic syndrome or TD2M and no changes in blood levels of glucose and HbA1c were recognized, suggesting that the profiling of miRNAs may support in either predicting the development of diabetes or in checking dietary and pharmacological interventions efficacy.

Besides, circulating miR-130a and miR-195 were linked to elevated blood pressure (Karolina et al. 2012). Metabolic syndrome was also linked to changes in circulating miR 23a, miR 27a, miR 130, miR 195, miR 197, miR 320a, and miR 509-5p (Karolina et al. 2012; Deiuliis 2016). Furthermore, cmiRNA profiles revealed a sex-specific link with metabolic syndrome (Wang et al. 2013). Yet, it has been suggested that let-7b, miR-143, and miR-221 are involved in both atherogenic and adipogenic processes (Hulsmans et al. 2011).

In morbidly obese patients, Ortega et al. discovered a significant increase in circulating miR-140-5p, miR-142-3p, and miR-222, as well as a decrease in miR-532-5p, miR-125b, miR-130b, miR-221, miR-15a, miR-423-5p, and miR-520c-3p. Weight loss due to surgery resulted in a significant diminution in circulating miR-140-5p, miR-122, miR-193a-5p, and miR-16-1, as well as an increase in miR-221 and miR-199a-3p levels according to the same study (Ortega et al. 2013). Likewise, levels of circulating miR-17-5p and miR-132 were lower in obese patients, mirroring the pattern of miRNA expression in omental fat within the same obese group (Heneghan et al. 2011).

Different studies have also reported that overweight/obese children and adolescents have a distinct cmiRNA signature than normal-weight children (Iacomino et al. 2016; Can et al. 2016; Prats-Puig et al. 2013; Thompson et al. 2017; Iacomino et al. 2019; Iacomino and Siani 2017). As an example, our team reported that a panel of cmiRNAs is differentially expressed in overweight/obese European children, with miR-551a and miR-501-5p upregulated and miR-10b-5p, miR-191-3p, miR-215-5p, and miR-874-3p downregulated, implying that these molecules may be useful in the

early detection of children at risk of excess body fat accumulation and connected metabolic anomalies (Iacomino et al. 2019). Two cmiRNAs, miR-122 and miR-34a, were found to be overexpressed in obese children with nonalcoholic fatty liver disease (NAFLD) and/or insulin resistance in a subsequent study (Oses et al. 2019).

Likewise, interestingly indications were found in newborns, pre-gestational maternal obesity, and gestational obesity (Carreras-Badosa et al. 2015). In babies born to obese or lean mothers, the expression of cmiRNAs, including miR-155, miR-181a, and miR-221, vary significantly. Such miRNAs were also proposed as tools predictor of obesity and risk of developing metabolic diseases in children born to obese mothers (Mendez-Mancilla et al. 2018).

Besides, multiple cmiRNAs were discovered to be changed in pre-gestational and gestational obesity with some linked to pregnancy weight gain, while others linked to metabolic parameters during pregnancy, and were predictors of pre and post-natal growth (Carreras-Badosa et al. 2015). Furthermore, various cmiRNA levels have been linked to gestational diabetes, particularly in pre-pregnancy overweight women (Wander et al. 2017).

Yet, recent research found that the miRNAs profile in maternal milk was also altered in overweight/obese lactating mothers, proposing that miRNAs could be implicated in a molecular communication between mother and newborn (Zamanillo et al. 2019).

Additional researchers focus on how nutrition, exercise, weight loss, and bariatric surgery affect miRNA profiles in obese patients. After a low or high glycemic index diet and a low-fat diet, individual cmiRNAs were shown to be significantly modulated in overweight/obese patients. Accordingly, the authors proposed cmiRNAs as innovative biomarkers in evaluating low-carbohydrate nutritional intervention (Giardina et al. 2019).

Furthermore, after acute aerobic activity, circulating miR-21, miR-126, miR-130b, miR-221, and miR-222 be increased in both obese and normal-weight patients (Bao et al. 2018).

Various studies have also demonstrated that gastric bypass has a significant impact on cmiRNA levels, with time-dependent modifications (Alkandari et al. 2018). The combination of gastric bypass surgery and exercise similarly changed the expression pattern of several cmiRNAs, confirming there are prospective novel biomarkers (Nunez Lopez et al. 2017). In this context, a recently published meta-analysis summarized different bariatric surgery results (Langi et al. 2019).

Applications to Prognosis, Other Diseases, or Conditions

The bulk of the 126,852 papers focused on miRNAs currently listed in PubMed (September 2021) shows a direct link to human diseases. A growing body of evidence supports the importance of miRNAs as prospective health-related tools (Keller and Meese 2016), with the majority of these studies focusing on "circulating" miRNAs as cancer theranostic biomarkers (Calin and Croce 2006; Matsuzaki and Ochiya 2017; Torres et al. 2019; Galvao-Lima et al. 2021). Actually, many reports

have established that human cancers frequently show aberrant expression profiles of miRNAs as well as pre-miRNAs. Of note, miRNAs can act as tumor suppressors or as onco-miRs (allowing the abnormal cell proliferation) which are connected to angiogenesis and tumor proliferation; besides, several miRNAs are involved in the control of cell cycle, induction of apoptotic mechanisms, and response to hypoxia and stress conditions. However, at present, there is a lack of consensus on specific miRNA panels to early detect cancer cells in vivo. Similarly, numerous miRNAs have also been reported for personalized medicine in infectious disease treatment (Tribolet et al. 2020) and other conditions.

Recent clinical trial data have predicted both anti-miR and miR-mimics compounds, as a new class of drugs for therapeutic applications in next-generation medicine, and numerous biopharmaceuticals companies are currently involved in this business (Chakraborty et al. 2021).

Mini-Dictionary of Terms

- Anti-miRs are single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs molecule.
- Circulating miRNAs are miRNAs packaged and secreted from cells arranged either into protein or exosomes complexes in several body fluids.
- Epigenetics is typically described as a heritable change that occurs in the DNA without affecting its sequence and includes post-translational modifications of core histones comprising acetylation, phosphorylation, methylation, and ubiquitylation.
- miRNA mimics are double-stranded RNA molecules designed to mimic endogenous miRNAs, resulting in down-regulation of target mRNA translation.
- miRNAs are short non-coding RNAs, 20–24 nucleotides in length, acting as post-transcriptional regulators of gene expression by base-pairing with target mRNAs.
- Short non-coding RNAs are classified as small interfering RNAs, PIWI-interacting RNAs, endogenous small interfering RNAs, promoter-associated RNAs, small nucleolar RNAs, and miRNAs based on their working mechanism, production, and structure.
- The similarity in seed region has been used to group miRNAs into “families” with the capability to target common clusters of mRNA transcripts.

Summary Points

- miRNAs play a relevant role in the development of obesity and obesity-related comorbidities by altering the status and activity of adipose tissue, pancreas, liver, and muscle.
- The fascinating emergence of circulating miRNAs as stable and affordable molecules has opened up a promising opportunity for the identification of

potentially useful non-invasive biomarkers for the early detection of subjects at higher risk of excess fat deposition and related metabolic abnormalities.
- Multiple candidate miRNAs have been discovered and clinical trials are ongoing to validate their relevance and impact.
- The use of these molecular biomarkers will support in either predicting the development of metabolic disease or in checking dietary and pharmacological interventions efficacy.
- Understanding the role of miRNAs in tissue metabolism and energy balance will help in the development of new biological targets and treatment methods for metabolic diseases.

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Plasma MicroRNA (miRNA)s as Novel Markers of Nonalcoholic Fatty Liver Disease

25

Implications for Diet and Nutrition

Ulas Emre Akbulut

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive fat build-up in the liver without any other evident causes of chronic liver disease (alcohol intake, genetic, viral, or autoimmune disease, etc.). NAFLD is currently the most common chronic liver disease worldwide due to the concomitant obesity pandemic. Both environmental factors and genetic predisposition contribute to the disease. Understanding of the pathophysiology of NAFLD has revealed that microRNAs (miRNAs), important epigenetic factors, may play significant roles in the disease pathogenesis. These molecules represent a class of small (19–25 nucleotides), noncoding, highly conserved endogenous RNAs

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that regulate gene expression at the post-transcriptional level. In this chapter, we summarize the current knowledge of miRNAs in lipid metabolism, insulin resistance, and oxidative stress, key factors that contribute to the occurrence and progression of NAFLD.

Keywords

Nonalcoholic fatty liver disease · Biomarkers · Epigenetics · MicroRNA · Nutrition · Diet · Insulin resistance · Fructose · Oxidative stress · Metabolism

Abbreviations

3′ UTR	3′-terminal untranslated region
ALT	Alanine aminotransferase
AMPKa	Activity of the protein kinase a
ChREBP	Carbohydrate response element binding protein
DGCR8	DiGeorge Syndrome Critical Region 8
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FAO	Fatty acid oxidation
FFA	Free fatty acid
FGF	Fibroblast growth factor
G6Pase	Glucose-6-phosphatase
GYS1	Glycogen synthase 1
HBP1D	HMG-box transcription factor 1D
HDL	High density lipoprotein
HMGCR	3-hydroxy-3-methyl glutaryl coenzyme A reductase
HNF	Hepatocyte nuclear factor
HNF	Hepatocyte nuclear factor
IR	Insulin resistance
JNK	Jun N-terminal kinase
MEG3	Maternally expressed gene 3
MiRNAs	Micro RNAs
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acids
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
Nrf2	Nuclear factor-erythroid 2-related factor 2
PCK	Phosphoenolpyruvate carboxykinase
PNPLA3	Patatin like phospholipase domain containing 3
PPARα	Peroxisome proliferator activated receptor alpha
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog

PTPN1	Protein tyrosine phosphatase non-receptor type 1
PUFA	Polyunsaturated fatty acids
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
SFA	Saturated fatty acids
SIRT	Specifically targets sirtuin
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element-binding protein
T2D	Type 2 diabetes
UPR	Unfolded protein response
VLDL	Very-low density lipoprotein

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by Wilson's disease, alpha-1 antitrypsin deficiency, viral hepatitis, metabolic diseases, and fat deposition exceeding 5% without significant alcohol consumption (Nuno-Lámbarri et al. 2016). NAFLD is a general term also including fatty liver, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. It is one of the most common liver diseases in both adults and children. The majority of patients with NAFLD are asymptomatic and are identified incidentally through laboratory findings or following imaging. A recent prospective cohort study involving ultrasonography and liver biopsy in asymptomatic, middle-aged patients reported prevalences of 46% and 12.2% for NAFLD and NASH, retrospectively (Vernon et al. 2011; Williams et al. 2011).

This complex clinical-pathological condition derives from genetic, environmental, and behavioral factors. Since increased body mass index and visceral adipose tissue deposition play a critical role in the development of NAFLD, obesity is a well-known risk factor for the disease. Several studies have shown that the frequency and severity of NAFLD is proportional to the degree of obesity. There is a close relationship between insulin resistance (IR), type 2 diabetes (T2D), dyslipidemia, and NAFLD (Diehl and Day 2017). IR causes fat deposition in the liver and is of critical importance in the pathogenesis of NAFLD. IR and T2D are present in as many as 70% of adult patients with NAFLD. Another important finding seen in patients with NAFLD is dyslipidemia, found in up to 80% (Stevanović et al. 2020).

Numerous studies have shown that specific macronutrients play a role in the development and progression of NAFLD. The macronutrient component of diet is associated with NAFLD/NASH independently of energy intake. Macronutrients such as saturated fatty acids (SFAs), trans fats, simple sugars (sucrose and fructose), and animal proteins are deleterious to the liver. These modulate triglyceride deposition and antioxidant activity in the liver, and this affects insulin sensitivity and postprandial triglyceride metabolism (Berná and Romero-Gomez 2020). In contrast, monounsaturated fatty acids (MUFAs), ω3 polyunsaturated fatty acids (PUFAs), plant-based proteins, and dietary fiber are beneficial to the liver (Perdomo et al. 2019).

Ethnic origin is an important risk factor for the development of NAFLD. One meta-analysis showed that the highest prevalence is in the Middle East and South America, and the lowest in Africa (Younossi et al. 2016). Male gender is another important risk factor for NAFLD (Younossi et al. 2018). Genetic and epigenetic modifications also play an important role in the development of NAFLD. For example, the patatin-like phospholipase domain-containing 3 (PNPLA3) gene is associated with fat deposition in hepatocytes and forms of NAFLD with poor prognosis. rs738409 single nucleotide polymorphism (SNP) in this gene (M148I) is one of the best-known SNPs and is associated with portal and lobular inflammation, the appearance of Mallory-Denk bodies, and fibrosis development (Dai et al. 2019b). Epigenetics, on the other hand, refer to inherited changes in deoxyribonucleic acid (DNA) and histones that do not cause changes in the nucleotide structure of DNA, but also do not contain SNPs, that are capable of being transferred by mitosis and/or meiosis, and that affect gene expression and phenotype. The best-known epigenetic mechanisms include micro-RNAs (miRNAs), DNA methylation, and histone modification.

MiRNAs are small (20–22 nucleotide), noncoding, highly conserved endogenous RNAs that regulate gene expression at the post-transcription level. The expression of the majority of mammalian genes has been shown to be regulated by miRNAs. These molecules play important roles in several physiological processes, such as cell growth, embryonic development, and apoptosis. Many miRNA are highly important regulators of hepatic functions including liver regeneration, lipid metabolism, apoptosis, and tissue development (Kerr et al. 2011; Lakner et al. 2011). In addition, several studies have shown dysregulation and modulation of miRNAs in NAFLD (Dongiovanni et al. 2018).

MiRNA Biogenesis

MiRNA genes encoded in the genome of nucleated cells are largely transcribed by RNA polymerase II to produce "primary miRNA (pri-miRNA)" (Borchert et al. 2006). Following transcription, Drosha, an RNA polymerase III enzyme, and its cofactor, the DiGeorge Syndrome Critical Region 8 (DGCR8), give rise to a large protein structure known as the microprocessor complex. This microprocessor complex enables the formation of "precursor miRNA" (pre-miRNA) approximately 70 nucleotides in length from pri-miRNA. The pre-miRNA that enters the cytoplasm with Exportin 5 present in the nuclear membrane is processed by an RNA polymerase III enzyme, Dicer, to form a mature miRNA pair approximately 22 nucleotides long (Yi et al. 2003; Lee et al. 2004). One strand of this double-stranded RNA molecule is broken down, while the other combines with the Argonaut protein complex to form RNA-induced silencing complex (RISC) (Lin and Gregory 2015). RISC directs single-stranded mature miRNA to attach to the "3′-terminal untranslated region (3′ UTR)" of mRNA (Bartel 2009).

MiRNAs regulate the gene expression of various cellular processes including differentiation, invasion, and cell death by binding to an mRNA responsible for inhibiting the translation of a protein or for inducing its degradation (Butt et al. 2016).

They essentially inhibit the protein translation of target genes (Choo et al. 2014). Our knowledge of the number of human genes regulated by miRNAs is increasing all the time. The implication of these molecules in various diseases, particularly those exposed to environmental factors, has attracted the interest of researchers. The genes targeted by miRNAs can be monogenic or polygenic (multifunctional). Indeed, more than one miRNA can target a single gene. These findings show that they possess a significant regulatory capacity.

MiRNA-Dependent Epigenetic Reprogramming in NAFLD

Various irregularly functioning metabolic pathways in NAFLD lead to abnormal lipid accumulation in hepatocytes. The most important of these are increased de novo lipogenesis (DNL), excess uptake of lipids present in blood, decreased hepatic lipid release (export), or impaired lipid oxidation (Samuel and Shulman 2018; Postic and Girard 2008). All these metabolic processes are regulated by miRNAs (Fig. 1). Changes in hepatic glycolysis, gluconeogenesis, and glycogen metabolism are also basic pathological mechanisms contributing to the development of NAFLD. miRNAs also pay a critical role in the control of these pathways. In addition, cellular processes such as endoplasmic reticulum (ER) stress, shown to be controlled by miRNAs, also play a role in the development of steatosis (Han and Kaufman 2016).

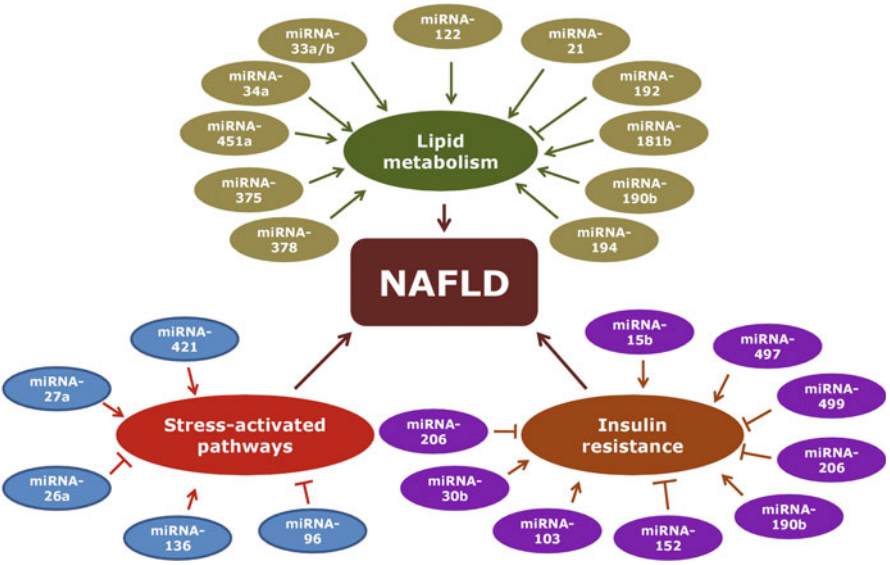

Fig. 1 Role of microRNAs in the pathogenesis of NAFLD

Hepatic Lipid Metabolism

The liver is an important organ for lipid metabolism. In case of lipid metabolic impairment, excessive fat accumulates in the liver, particularly in hepatocytes, leading to the development of NAFLD. Lipid metabolism is affected by various processes, particularly DNL, excessive fat intake, fatty acid oxidation (FAO), and very low-density lipoprotein (VLDL) export. In case of impairment of the functioning of these processes, hepatic lipid metabolism is compromised, and excessive hepatic triglyceride accumulation occurs (Liu et al. 2015a, b). Excessive accumulation of triglycerides makes the liver susceptible to proinflammatory cytokine attacks, resulting in mitochondrial dysfunction and oxidative stress. Hepatic steatosis then develops in consequence. SFA is associated with increased adipose tissue deposition in the liver for reasons such as increased DNL in the liver, and increased lipolysis in fatty tissue. In contrast, increased unsaturated fat intake is associated with decreased lipolysis by preventing fat tissue deposition in the liver (Rosqvist et al. 2019). An SFA diet is also associated with increased oxidative stress leading to impaired glutathione metabolism and progression of NAFLD (Franco et al. 2018). However, it is unclear whether different sources of SFA (such as milk and meat) have different effects on hepatic fat tissue. The fact that the effects of saturated fats on the liver depend on the patient's genetic history should not be overlooked.

MiRNA-122 is the most investigated miRNA, and represents 70% of total miRNAs. It is involved in hepatocyte proliferation and maturation by stimulating the expression of 24 specific genes, including hepatocyte nuclear factor 6 (HNF6) (Pirola et al. 2015; Thomas and Deiters 2013). It also reacts with numerous target genes involved in lipid and cholesterol metabolism. Circulating miRNA-122 levels increase significantly in patients with NASH, and the hepatic expression of miRNA-122 decreases, which also shows that miRNA-122 in serum is secreted by hepatocytes (Pirola et al. 2015; Cheung et al. 2008; Miyaaki et al. 2014). Studies have shown that circulating miRNA-122 levels are correlated with alanine aminotransferase (ALT) levels in patients with NASH, and can indicate the severity of NAFLD better than classic hepatic function markers (Pirola et al. 2015; Becker et al. 2015). Mouse studies have shown that inhibition of miRNA-122 reduces hepatic fatty acid and cholesterol synthesis, as well as reducing hepatic fatty acid oxidation, which reduces plasma cholesterol levels (Esau et al. 2006; Krützfeldt et al. 2005). SFAs increase circulating miRNA-122 and reduce its levels in hepatocytes (Chai et al. 2017).

MiRNA-33a and miRNA-33b are copied together (co-transcribed) with sterol regulatory element-binding protein 1 (SREBP1) and SREBP2, regulators of DNL and cholesterol biosynthesis. They reduce high density lipoprotein (HDL) synthesis by suppressing ATP-binding cassette transporter member 1. They also contribute to the modulation of fatty acid metabolism and to the regulation of cholesterol and insulin synthesis (Vega-Badillo et al. 2016). This increases the inhibition of miRNAs, insulin sensitivity, beta oxidation, and circulating HDL levels (Davalos et al. 2011; Rayner et al. 2011; Li et al. 2014).

MiRNA-21 irregularity in several human tissues is associated with cancer, inflammation, fibrosis, and NAFLD (Wang et al. 2021; Benhamouche-Trouillet and Postic

2016). MiRNA-21 levels in circulation and expression in the liver have been shown to increase in patients with NAFLD and in mouse models (Benhamouche-Trouillet and Postic 2016; Pillai et al. 2020; Liu et al. 2018). MiRNA-21 regulates triglyceride, free cholesterol, and total cholesterol levels through 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR) inhibition and fatty acid binding protein 7 (FABP7) mediation. In addition, it contributes to the development of NAFLD through the mediation of such targets as SMAD7, phosphate and tension homolog (PTEN), and HMG-box transcription factor 1D (HBP1D) (Ahn et al. 2012; Sun et al. 2015; Loyer et al. 2016; Dattaroy et al. 2015). This miRNA is a typical representative of stress-induced miRNAs that are powerfully expressed in the liver but that remain inactivated under normal physiological conditions (Calo et al. 2016).

Although miRNA-34a expression in hepatocytes is high, it can also be closely associated with lipid metabolism. Human and mouse studies have shown that miRNA-34a specifically targets sirtuin 1 (SIRT1) and peroxisome proliferator activated receptor alpha (PPARα). Fatty acid catabolism is restricted with MiRNA-34a inhibition, and steatosis develops (Ding et al. 2015). miRNA-34a inhibition also increases the activity of the protein kinase a (AMPKa) activated with AMP, and lipogenesis is antagonized as a result. MiRNA-34a has also been shown to exert control over lipid storage in mice by specifically targeting hepatocyte nuclear factor 4 (HNF4), which inhibits the development of steatosis through the transcriptional control of various genes involved in lipid catabolism (Xu et al. 2015b).

Decreased expression of miRNA-192 and upregulation of SREBP1 have been reported in mice given bisphenol A, as a result of which steatosis and lipid accumulation in hepatocytes has been detected (Lin et al. 2017). miRNA-451a also regulates the expression of thyroid hormone response protein 14, which plays an important role as a negative regulator of DNL (Zeng et al. 2018). Additionally, miRNA-375, miRNA-378, miRNA-181b, miRNA-190b, and miRNA-194 by affecting DNL (Zhang et al. 2018; Lei et al. 2018; Wang et al. 2017a, b; Xu et al. 2018; Nie et al. 2017). Some of the miRNAs with irregular expression in liver tissues or abnormalities in the systemic circulation are shown in Table 1.

In conclusion, the serum levels of various miRNAs such as miRNA-122 and miRNA-21 differ significantly between patients with NAFLD/NASH and healthy controls and possess the potential for use as a noninvasive biomarker. MiRNAs are involved in all stages of lipid metabolism, including DNL, fatty acid oxidation, lipid transport, and cholesterol metabolism. Lipid accumulation is regarded as the "first hit" in NAFLD. Amelioration of steatosis is of considerable importance for blocking the progress of NAFLD, and the miRNAs described above can provide a theoretical basis for miRNA-based treatment.

Hepatic Carbohydrate Metabolism

Carbohydrates are classified as monosaccharides (fructose, glucose, and galactose) and disaccharides. Fructose is naturally present in fruits, and is also contained, generally in the form of additional sugar, and as a sweetener in the form of saccharose

Table 1 Deregulated miRNAs in liver tissues and systemic circulation of NAFLD patients

MicroRNA	Circulation level	Liver expression	References (PMID)
miRNA-122	↓	↑	19030170, 24973316, 26565986, 29848284
miRNA-21	↑	↑	29523084, 27222533, 26338827
miRNA-34a	↑	↑	32933141, 29523084, 26330104, 26100857
miRNA-27a	↓	↑	31889412, 31827150
miRNA-192	↑	↑	29523084, 24973316
miRNA-29a	↓	↓	29848284, 31652636
miRNA-206	Unknown	↓	28025059
miRNA-152	Unknown	↓	26996529
miRNA-147	Unknown	↑	26300412
miRNA-149	Unknown	↓	26279439
miRNA-30b	↑	↑	30721562
miRNA-15b	↑	↑	26179126, 23287814
miRNA-421	Unknown	↑	27107702
miRNA-26a	Unknown	↓	33548387

(50% glucose and 50% fructose) in other products such as alcohol-free beverages, biscuits, and bakery products goods. The adverse metabolic effects of excessive consumption of simple carbohydrates have long been known. In addition, there are suspicions concerning the role in the development of NAFLD of monosaccharides and disaccharides in foodstuffs. In contrast, epidemiological studies have yielded no convincing evidence of a link between additional sugars (saccharose, fructose, and high-fructose corn syrup) and NAFLD (Howard and Wylie-Rosett 2002).

A positive association exists between the risk of NAFLD development and high-fructose products (cakes, alcohol-free beverages, and sugary snacks) (Perdomo et al. 2019). Controlled studies have shown that fructose intake exceeding 25% of energy requirements has deleterious effects on insulin sensitivity and intrahepatic triglycerides (Softic et al. 2016; Stanhope et al. 2009). Fructose stimulates DNL, inhibits hepatic lipid oxidation by blocking PPARα activity, and increases fibroblast growth factor 21 (FGF21) levels in a carbohydrate response element binding protein (ChREBP)-dependent manner even when protein intake is controlled (Roglans et al. 2007; Iroz et al. 2017; Lundsgaard et al. 2017). Fructose also activates c-Jun N-terminal kinase (JNK) and nitro-oxidative stress marker cytochrome P450-2E1 (CYP2E1). Fructose-derived biochemical changes lead to obesity, fat deposition, insulin resistance, inflammation, and hepatic fibrosis (Wei and Pagliassotti 2004; Cho et al. 2021).

Hepatic glucose uptake and use decrease in IR. In order to compensate for rising blood sugar levels, the body produces and releases large quantities of insulin, a phenomenon known as hyperinsulinemia (Bugianesi et al. 2010). Numerous studies show that both genetic and environmental factors are associated with IR development. Obesity and hyperglycemia in particular play a key role in the development of IR (Buzzetti et al. 2016). IR increases DNL by causing excessive sterol regulatory element-binding protein 1 SRBEP1 expression and accelerates adipose tissue

lipolysis, and thus plays a critical role in the pathogenesis of NAFLD by leading to excessive fatty acid deposition in the liver (Anstee and Day 2013; Bugianesi et al. 2010). Excessive SREBP1 expression positively regulated the proapoptotic molecule Fas. Fas has been implicated in the development of NAFLD by inducing IR and hepatic steatosis (Liu et al. 2015a, b; MacHado and Diehl 2016). IR also adversely affects adipose tissue function, as a result of which the normal regulation of inflammatory cytokines and adipokines is impaired (Guilherme et al. 2008).

Carbohydrate and lipid metabolism pathways are connected through shared biochemical substrates and need to be considered together in the development of NAFLD. MiRNAs that regulate glucose metabolism in addition to IR have been reported. MiRNA-122 targets glycogen synthase 1 (GYS1) and limits glycogen synthesis (Esau et al. 2006). In addition, miRNA-122 has been shown to target glycolytic enzyme aldolase A in cells (Castoldi et al. 2011). This may suggest that miRNA-122 downregulation in NAFLD is responsible for the upregulation of glycolysis. MiRNA-29 blocks glucose uptake via insulin-stimulated Akt, by preventing insulin signaling (He et al. 2007). Upregulation of miRNA-29 in HepG2 cells leads to IR, one of the most important risk factors for the development of NAFLD (Pandey et al. 2011). Interestingly, miRNA-29a serum levels are significantly downregulated in patients with NAFLD (Jampoka et al. 2018). MiRNA-33 inhibits gluconeogenesis through the activation of phosphoenolpyruvate carboxykinase (PCK1) and glucose-6-phosphatase (G6Pase) in patients with insulin resistance and NAFLD (Ramírez et al. 2013). MiRNA-206 inhibits the protein tyrosine phosphatase non-receptor type 1 (PTPN1). Thus, it can improve insulin signaling and reduce hepatic lipogenesis (Wu et al. 2017). Hepatic expression of miRNA-152 was significantly downregulated in mice receiving a high-fat diet (Wang et al. 2016). This leads to impairment of hepatic glycogenesis through PTEN upregulation. The PI3K/PDK1/AKT pathway is an important factor in insulin signaling, and PI3K activity is regulated by PTEN. It exerts a similar effect on miRNA-499-5p through the same target as miRNA-152 (Wang et al. 2015b). High miRNA-497 levels can inactivate the IRS-1/PI3K/Akt/GSK-3b/GS pathway by inhibiting insulin receptor expression, and can thus improve hepatic IR (Wang et al. 2015a). MiRNA-15b exhibits the same effect via insulin receptor (Yang et al. 2015). In addition, miRNA-30b, miRNA-206, miRNA-190b, and miRNA-103 can contribute to the development of NAFLD by affecting IR (Xu et al. 2018; Dai et al. 2019a; Wu et al. 2017; Xu et al. 2015a).

Some miRNAs involved in the regulation of insulin signaling, and this lipid metabolism, have been briefly described above. IR is known to contribute to excessive lipid accumulation in the liver, and this results in NAFLD. Therapy focusing on insulin signaling may be useful for patients with NAFLD.

Stress-Activated Pathways

Redox homeostasis is established by careful regulation of both the generation of reactive oxygen species (ROS) and their removal from the body. It is of vital importance to the maintenance of normal cellular functions. Oxidative stress, the impairment of the regulatory role of ROS, can affect various physiological processes

and contribute to the pathogenesis of various diseases. ROS are defined as reactive oxidizing agents produced in the mitochondria, peroxisomes, and ER as a result of both enzymatic and nonenzymatic reactions (Gorrini et al. 2013; Engedal et al. 2018). Overproduction of ROS impairs normal physiological processes by damaging molecules such as DNA, proteins, and lipids. ER is associated with protein maturation. When excessive free fatty acid (FFA) reaches the liver, the ER in hepatocytes has to produce a greater quantity of protein. The results are a larger number of misfolded proteins, known as ER stress, and the activation of a protective program known as unfolded protein response (UPR). Continuous UPR activation contributes to the development of NAFLD by affecting lipid accumulation, mitochondrial activities, and insulin signaling (Liu et al. 2021). Excessive FFAs lead to ROS overproduction capable of damaging the mitochondria and leading to lipotoxicity in the progression of NAFLD (Koliaki et al. 2015). Lipotoxicity leads to ER stress, increasing inflammation, hepatocyte damage, and finally hepatocyte death. Interestingly, ER stress also encourages ROS production (Ashraf and Sheikh 2015). One study showed that miRNA26a upregulation was stimulated following ER stress-inducing therapy in NAFLD (Xu et al. 2021). miR-421 inhibits SIRT3 and thus affects normal mitochondrial functions in NAFLD. miR-421 expression has been found to increase significantly in mice with NAFLD, thus exacerbating the severity of oxidative stress and increasing lipid accumulation via the SIRT3/FOXO3 signaling pathway. Suppressing hepatic miR-421 is thought to be capable of ameliorating oxidative stress-associated cellular damage in NAFLD (Cheng et al. 2016). miRNA-26a expression in liver cells is induced by ER stress, and this ameliorates ER stress by targeting eIF2a. Overexpression of MiRNA-26a has been shown to improve ER stress and lipid accumulation both in vitro and in vivo. In contrast, miRNA-26a deficiency exacerbates ER stress and the progression of NAFLD (Xu et al. 2021). Nuclear factor-erythroid 2-related factor 2 (Nrf2), a key modulator of the cellular antioxidant system, cab also affect the pathogenesis of NAFLD. Nrf2 expression can be inhibited in case of overexpression of miRNA-27a, lading to increasing ROS production and lipid accumulation (Teimouri et al. 2020). In addition, miRNA-136 has been shown to increase Nrf2 and maternally expressed gene 3 (MEG3) levels, while miRNA-96-5p ameliorates NAFLD through inhibition of the p66shc/cytochrome C cascade (Wang and Wang 2018; Zhang et al. 2020).

In summary, oxidative stress and ER stress contribute to lipid accumulation and hepatocyte apoptosis, thus exacerbating the progression of NAFLD. Several miRNAs, such as MiRNA-26a, miRNA-27a, and miRNA-136, regulate the critical redox pathways. This information may provide clues to assist with monitor disease progression and the development of miRNA-targeted therapies in NAFLD.

MiRNAs That Regulate NAFLD Progression

Liver fibrosis is a pathological process in which extracellular matrix accumulates and damage repair continues. Fibrosis is an important indicator or mortality in NAFLD (Schuppan et al. 2018). Hepatocyte lipoapoptosis is the most important factor in the progression of fibrosis (Du et al. 2015).

One study reported that miRNA-122 levels were 7.2-fold higher in patients with NASH compared to those healthy individuals, and 3.1-fold higher than those of patients with simple steatosis. This suggests that miRNA-122 may represent a marker for NASH (Pirola et al. 2015). However, there are also studies reporting negative correlation between hepatic and serum miRNA-122 levels. Serum miRNA-122 levels exhibit positive correlation with severity of NAFLD in animal models, albeit without ALT elevation. Hepatic stellate cells are activated under pathological conditions, and these represent the main source of fibrogenic myofibroblasts. The multiplication and activation of hepatic stellate cells in NASH has been shown to be capable of suppression by miRNA-146a-5p (Du et al. 2015). In contrast, MiRNA-214 is markedly upregulated during hepatic stellate cell activation and contributes to liver fibrosis (Ma et al. 2018). However, miRNA-130a-3p suppresses hepatic stellate cells and ameliorates fibrosis in NASH (Wang et al. 2017a, b).

Contrary to belief, the incidence of malignancy in patients with NAFLD without cirrhosis increases (Baffy 2013). The molecular mechanisms associated with cancer development in the absence of cirrhosis remain largely unknown. However, it is thought that some non-genomic changes in NAFLD may create a microenvironment leading to hepatic tumorigenesis. Loss of expression/activity of PTEN, an important steatosis suppressant, has been shown with steatosis in both animal and human studies (Sanchez-Pareja et al. 2016; Vinciguerra et al. 2008). It has been suggested that miRNA-21 targets PTEN expression in hepatic cancer cells and it's a potent oncogenic miRNA (Meng et al. 2007). For that reason, in addition to its role in hepatic metabolism and NAFLD/NASH development, upregulation of miRNA-21 in NAFLD may also lead to tumor formation. In contrast to miRNA-21, miRNA-122 has been shown to play a tumor-suppressing role through mechanisms such as inhibition of the oncogene c-Myc and enhancing hepatocyte differentiation in the human liver (Wang et al. 2014; Coulouarn et al. 2009).

Applications to Prognosis, Other Diseases or Conditions

New and improved biomarkers are needed for rapid diagnosis of diseases with high mortality and morbidity rates. The most important features of a good biomarker are specificity, sensitivity, and stability, but also that they can be obtained in a relatively noninvasive manner. MiRNAs as potential biomarkers, since they are stable and can be detected in the blood, urine, or other body fluids using simple, sensitive, and relatively cheap assays, even after periods of years of sample storage. Using multiple protein markers can be both expensive and time-consuming. However, using multi-marker panels of multiple miRNAs may be a noninvasive method for diagnosing disease progression. Besides NAFLD, numerous miRNA levels are changed in diseases that are the main cause of morbidity and mortality, from cardiovascular disease to neurological disorders, rheumatic diseases, sepsis, and cancer. MiRNAs are released into the circulatory system from different organs such as the brain, heart, liver, ovary, uterus, and mammary glands in both healthy humans and patients. In addition, in cancer patients, some miRNAs are released from tumor tissues and can

be detected in the blood. After resection of the tumor, oncogenic miRNAs levels tend to decrease. Therefore, miRNAs can be used to determine tumor progression.

Nowadays, a large number of miRNA biomarkers have been reported, but the prospects for practical application are still unclear. Because, standard protocols for the collection, transport, storage, and analysis methods of miRNAs samples are not yet available. However, despite current limitations, miRNAs can be widely used as biomarkers as techniques improve.

Conclusion

Diet and lifestyle are responsible for the development of NAFLD. In particular, powerful data in the literature overemphasize the potentially negative role of n-6 PUFAs and SFAs in the development of liver diseases, as well as a diet rich in fructose and added sugars. There is currently no treatment for NAFLD, and lifestyle modifications aimed at weight reduction remain the sole therapy giving promising results. The elimination from daily diet of milk and animal fats in addition to sugar-containing beverages and bakery goods, and their replacement with complex carbohydrates, whole grains, fiber, olives, vegetable fats rich in n-3 PUFA and MUFA, fish, grains, and nuts is therefore recommended.

In addition to diet and lifestyle, genetic or epigenetic factors also play a role in the pathogenesis and progression of NAFLD. There has been increasing evidence in recent years of interaction between genetic disposition and environmental factors (especially diet) in the development of NAFLD, and the novel concept of nutritional genomics or nutrigenomics has emerged as a result.

Epigenetics involves inherited changes in gene expression or the cellular phenotype without altering the underlying DNA sequence. Epigenetics includes DNA methylation, chromatin variation, and noncoding RNAs, particularly miRNAs. miRNAs play a critical role in the post-transcriptional regulation of target genes involved in the pathogenesis of NAFLD/NASH. They perform these roles in the development of NAFLD through various mechanisms. Abnormal expression of miRNAs leads to deregulation of glucose and lipid metabolism contributing to the etiology of hepatic steatosis. Regulating the expression of these miRNAs may make it possible to reprogram abnormal metabolic pathways that lead to the disease. Serum miRNA profiling has been performed in different cohorts aimed at identifying circulating miRNAs potentially capable of use as biomarkers for the early diagnosis or follow-up of disease progression in patients with NAFLD.

Key Facts

Insulin resistance is known to contribute to excessive lipid accumulation in the liver, and this results in NAFLD.

Some miRNAs are involved in the regulation of insulin signaling, and in all stages of lipid metabolism, including de novo lipogenesis, fatty acid oxidation, lipid transport, and cholesterol metabolism.

The serum levels of various miRNAs such as miRNA-122 and miRNA-21 differ significantly between patients with NAFLD/NASH and healthy controls and possess the potential for use as a noninvasive biomarker.

Mini-Dictionary of Terms

Biomarkers. Substances produced by the body that can be detected in body fluids such as blood or urine and indicate a specific process, condition or disease.

MicroRNAs. MiRNA is the name given to molecules consisting of a single-stranded nucleotide sequence that was first discovered in 1993, but was named in 2001. These RNAs bind to their target mRNAs to inhibit protein production. In this way, they regulate the expression of the target gene by degrading mRNA or suppressing the translation process in the cell.

Pri- and pre-miRNA. MiRNAs are noncoding RNAs, meaning they are encoded by genes that are transcribed from DNA but not translated into protein. Primary transcripts, called pri-miRNA, are processed and transformed into structures called pre-miRNA and then into functional miRNA.

Summary Points

The most important risk factor in the development of NAFLD is insulin resistance due to obesity.

The prevalence of NAFLD is increasing due to the increasing prevalence of obesity in the world.

High consumption of saturated fatty acids and fructose is thought to contribute to the development of NAFLD.

In addition to environmental factors, genetic predisposition is also important for the occurrence of NAFLD.

Epigenetic mechanisms, in particular those associated with microRNAs, are associated with nonalcoholic fatty liver disease.

Aberrant expression of miRNAs contributes to deregulation of glucose and lipid metabolism, contributing to the etiology of diabetes and hepatic steatosis.

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Telomere Length

26

Measurement and Application as a Biological Indicator – Links with Anthropometry in Lifestyle Intervention

A. Marti del Moral and G. Zalba Goñi

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Abstract

Abnormal telomere shortening underlies patients' risk of developing age-related degenerative diseases, including metabolic and cardiovascular diseases, and cancers. The measurement of telomere length (TL) and the detection of excessively short telomeres emerge as an important new tool in biomedical research and clinical practice. The first section reviews the main methods for measuring telomere length and compares the technologies, pointing out their advantages and limitations. The second section provides information on the role of TL as a biomarker of response in lifestyle intervention aimed to reduce cardiometabolic risk in adult and pediatric populations. There is evidence that substantial weight loss is able to lower chronic inflammation and adipose tissue oxidative stress which can lead to promote TL conservation and DNA repair, thus reducing telomere attrition. Inconsistent results concerning the benefit of lifestyle intervention on TL suggest the need for more studies – probably devoted to the measurement of ***excessively short telomeres*** – before its clinical application in routine use as biomarker.

Keywords

Short telomere · High throughput · Obesity · Anthropometric indexes · Dietary intervention · Lifestyle changes · Telomere length · Pediatric population · Mediterranean diet

Abbreviations

BMI	Body mass index
BMI-SDS	Body mass index standard deviation score
bp	Base pairs
MD	Mediterranean diet
MET	Metabolic equivalent
MMqPCR	Monochrome multiplex qPCR
PA	Physical activity
PCR	Polymerase chain reaction
PPARG2	Peroxisome Proliferator Activated Receptor Gamma 2
Q-FISH	Quantitative Fluorescence in situ Hybridization
qPCR	Quantitative PCR
STAR	Single telomere absolute-length rapid
STELA	Single telomere length analysis
TL	Telomere length
TRF	Terminal restriction fragment
T/S ratio	Telomere/Single copy gene ratio
WHO	World Health Organization

Introduction

Abnormal telomere shortening increases the susceptibility of patients to develop degenerative diseases associated with age, diabetes mellitus, cardiovascular diseases, and cancers. The first section reviews the main methods for measuring telomere length and compares the technologies, pointing out their advantages and limitations. The second section provides information on the role of TL as a biomarker of response in lifestyle intervention aimed to reduce cardiometabolic risk in adult and pediatric populations.

Section 1. Telomere Length: Measurements

Telomeres are regions of repeating noncoding nucleotide sequences (5′-TTAGGG-3′ in humans) at the end of each chromosome. Their main function is to protect the ends of the chromosomes from degradation and maintain the integrity of the chromosomes. The size of human telomeres ranges between 8 and 15 kb after birth, and decreases around 150 bp with each replication. The gradual shortening of telomeres causes them to reach a minimum critical length, which causes the cell to enter a state of senescence.

Abnormal telomere shortening increases the susceptibility of patients to develop degenerative diseases associated with age, diabetes mellitus, cardiovascular diseases, and cancers. In this context, the measurement of telomere length (TL) has experienced growing interest in the field of biomedicine, due to its potential use as a biomarker that can be used both in the diagnosis of diseases associated with age and different cancers, as well as in the response of patients to various therapies to treat these pathologies. Despite this, at present there are still significant technical challenges that limit its use as a daily tool in clinical practice. In order to solve this deficiency, the ideal method for the determination of TL should be simple, precise, and reproducible in any biomedical research laboratory, and fast and high throughput.

In this section, we will review the most used methods to measure TL, comparing them and discussing their advantages and weaknesses (Table 1). Specifically, we will focus on the following methods (Fig. 1):

- Terminal restriction fragments analysis, the gold standard for telomere determination, involves a Southern blot procedure of restriction fragments of genomic DNA.
- Quantitative polymerase chain reaction (PCR), a procedure in which the quantification of telomeres are referred to a single copy gene.
- Quantitative fluorescence in situ hybridization (FISH) approaches, in which the measurement involves the use of digital fluorescence microscopy.
- Single telomere length analysis (STELA), a method based on PCR, after the ligation of a linker to the ends of the chromosomes.
- Single telomere absolute-length rapid (STAR) assay, a novel digital real-time PCR approach.

Table 1 Comparative characteristics of TL measurement methods

Method	Applicability	Amount of starting material	Processing time	Labor intensive	Throughput	Dynamic range	Telomere length distribution	Absolute length
TRF	Research and commercial	> 1 μg	2 days	Yes	32 samples	0.8–20 kb	Yes (semi-quantitative)	Yes
qPCR	Research	10–20 ng	<2 h	No	384 samples	Measures relative length	No	No
Metaphase Q-FISH	Research	20 cells	2 days	Yes	10 samples per week	0.15–80 kb	Yes	Yes
High-throughput interphase Q-FISH	Research and commercial	10,000 cells	2 days	Yes	96 samples	0.2–80 kb	Yes	Yes
STELA, U-STELA	Research	50–200 pg	2 days	Yes	32 samples	0.4–20 kb	Yes, for short telomeres	Yes
STAR	Research	< 1 ng	<3 h	No	48 samples	0.2–320 kb	Yes	Yes

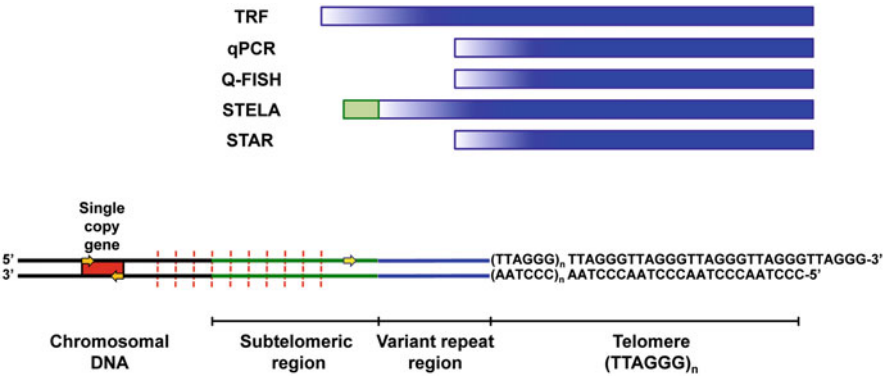

Fig. 1 Representation of chromosomal ends and the procedures for telomere length estimation. Telomeres are represented with the TTAGGG and AATCCC sequence repeat, the variant region (composed of degenerate TTAGGG repeats and telomere associated repeats) is represented by a thick blue line, the subtelomeric region is shown as a thick green line, and the rest of chromosomal DNA is reproduced as a thick black line where a single copy gene (red box) can be amplified by PCR using specific primer (orange arrows). Frequent cutting restriction endonucleases are also illustrated (red dotted lines). The telomere length estimation of the used techniques is represented as the blue colored boxes on the top: TRF includes part of the variant repeat region and subtelomeric region which are not digested by the frequent cutting restriction endonucleases. qPCR methods compare the amplification of telomeric DNA with the amplification of a single copy gene (red box) and can include part of the variant repeat region. Q-FISH probes recognize pure telomeric repeats but tandems of these can be also part of the variant repeat region. STELA uses a specific primer of the subtelomeric region (yellow arrow), but since this region is well characterized it can be subtracted to the telomere length quantification. STAR measures by PCR the absolute lengths and quantities of individual telomeres, after digestion of genomic DNA with restriction endonucleases

Terminal Restriction Fragments (TRF)

TRF analysis was the first technique used to measure TL and is known as the "gold standard" in telomere quantification (Montpetit et al. 2014). This approach is used to calibrate and validate other methods. The procedure begins when genomic DNA is digested with a combination of four base-pair restriction enzymes. These enzymes do not cut telomeres due to the repetitive nature of this sequence (TTAGGG)n, so the telomeric DNA remains intact; the rest of the chromosomal DNA is cut into small fragments (<800 bp). Electrophoresis of the digested genomic DNA and subsequent analysis by Southern blot allows the detection of uncut telomeric fragments, using a radioactive or nonradioactive (TTAGGG)n-labeled probe (Kimura et al. 2010). Each sample will present a smear that represents all the telomeric fragments, the result of the integration of the differences in the length of the 96 telomeres present in each cell, and the differences among the different cells that make up the sample. After quantifying the intensity of the labeled DNA smear and comparing with a DNA ladder with known fragment sizes, it is possible to infer the average.

TRF analysis is a method accurate and with relatively small coefficient of variation that does not require costly or specialized equipment, which provides an

absolute quantification in kilobases of average TL. Since it is not chromosome specific, the result obtained represents the mean TL of the whole simple. Moreover, it is a labor intensive and time-consuming procedure (5–7 days) that requires a great amount of starting DNA (1 μg), thus conditioning its application preferentially to TL determination in blood samples rather than in other tissues. In addition to the characteristic canonical sequence of telomeres, they also contain a subtelomeric region consisting of non-canonical, degenerate telomere repeats, whose length may vary depending on the endonuclease cocktail used. Thus, TRF analysis may overestimate the TL that explains the high variability of results found among different research laboratories. A last disadvantage is its low sensitivity to detect short telomeres (2 kb or less), which have few TTAGGG tandem sequences. Short telomeres are a characteristic of diseases related to senescence and aging, so the inability to detect them represents a significant lack of information (Vera and Blasco 2012).

Some of these limitations can be minimized by quantifying the telomeres of specific chromosomes after sorting by flow cytometry, by using pulsed-field electrophoresis that increases the resolution of the gel, or by reducing the necessary starting amount of genomic DNA (TRF slot) (Marti and Zalba 2017).

Quantitative PCR Methods

Quantitative PCR (qPCR) methods are easy assays that allow the quantification of TL in a small amount of DNA (ng or less). They are rapid, relatively low-cost methods that do not require the use of expensive or specialized equipment and are easily suitable for high-throughput performance, which has led to their use in genetic and epidemiological studies (Cawthon 2002). With this strategy, the canonical sequences of telomeres are specifically counted, without producing interferences derived from the subtelomeric regions.

Due to the repetition of the TTAGGG sequence along the telomeres, the first generation primers used to amplify them were complementary to each other, which favored the formation of primer dimers instead of amplifying the target DNA sequence. This complication was partially saved by the design of primers that bound to the C- and G- regions, but were mismatched at the other bases (Cawthon 2009). In this approach, the telomere (T) measurement is normalized against the measurement of a reference single copy gene (S), which provides a T/S ratio that acts as a relative index of the mean length of telomeres. The amplifications of T and S are carried out in separate tubes, which compromise the precision of the determination.

In an updated approach, both the T and S measurements could be performed within the same tube (Cawthon 2009). In this case, simultaneous amplification generates two amplicons that have relevant differences that allow them to be identified and quantified separately within the same tube. The telomeric amplicon has a relatively low melting temperature (≈81 °C) and is quantified in earlier cycles of the reaction, when the amplicon levels of the single copy gene are not yet detectable. On the other hand, the single copy amplicon has a melting temperature

(≈91 °C) higher than the telomeric one, and it is quantified in subsequent cycles and at a temperature higher than that used for the quantification of the telomeric amplicon. The increase in the quantification temperature allows to measure only the fluorescence generated by single copy amplicon, avoiding interference from the much more abundant telomeric amplicon signal. This method reduces operator-generated variability (pipetting errors), is more economical, and correlates better with other methodological approaches.

Although initially the PCR methods only provided a relative measure of telomeres (the T/S ratio), and not an absolute value (kilobases), this limitation can be solved if a standard curve of telomeric fragments of known length is used in the procedure (Montpetit et al. 2014). Similar to TRF analysis, this methodology only provides the mean TL of a sample; it cannot quantify the length of telomeres in a single cell or a specific chromosome, and it is unable to detect the presence of short telomeres.

A major shortcoming of qPCR approaches is the high variability between determinations made by different laboratories, which limits comparisons between different studies. This may be because the intra- and inter-assay coefficients of variation of qPCR are higher than those found with other procedures (Aviv et al. 2011; Lindrose et al. 2021). This may probably be associated with the absence of standardized protocols (use of different reagents and PCR machines or different single copy genes), so that their optimization could result in a substantial decrease in these variabilities, as has already been suggested by some researchers (Pejenaute and Zalba 2017).

The applicability of qPCR is limited to samples that are normal diploid, and its use is not suitable in cancer studies in which the reference single copy gene may have been duplicated or lost due to aneuploidy, alterations that occur in the majority cancer cells (Lai et al. 2018).

Quantitative FISH

The Quantitative Fluorescence in situ Hybridization (Q-FISH)) technology is based on the hybridization of a fluorescent probe complementary to the TTAGGG repeats sequence of telomeres (Lansdorp et al. 1996; Rufer et al. 1998; Dweck and Maitra 2021). Each probe binds to three TTAGGG telomeric repeats so there is a direct proportion between fluorescence intensity and TL, which can be detect and quantify by digital fluorescence microscopy (Metaphase and Interphase Q-FISH) or flow cytometry (Flow-FISH). A synthetic peptide nucleic acid molecule is mostly used as the fluorescent probe rather than the classical oligonucleotide probe. The probe only binds to the perfect TTAGGG telomeric repeats, so partial hybridizations with the non-canonical repeats of the subtelomeric region cannot occur. However, this probe can also recognize tandems of TTAGGG repeats in intrachromosomal regions, which may generate some false-positive results and produce the consequent overestimation of TL. Q-FISH assays are performed directly in cells which could be fresh, frozen, fixed, or embedded in paraffin, which allow to determine TL of a single

cell (Flow-FISH and Interphase Q-FISH), as well as the telomeric end of a single chromosome (Metaphase Q-FISH). Interestingly, Q-FISH has a low detection limit, which makes it suitable for quantifying critically short telomeres. In fact, this technique made it possible to demonstrate that cell viability and chromosomal stability critically depended on the presence of the shortest telomeres, and not on their average length.

In **metaphase Q-FISH**, the cells are stopped in metaphase and spread on a slide, after which the DNA is fixed and denatured to proceed to hybridize the telomeric DNA with the fluorescent probe. Finally, the images are visualized and acquired with a fluorescence microscope or with a digital imaging system that allows quantifying the fluorescent signals of the telomeres, which are finally transformed into kb values, thanks to the use of control samples of known length (Lansdorp et al. 1996; Pejenaute and Zalba 2017). Metaphase Q-FISH can determine the size of each telomere from a specific chromosome, with a very precise and sensitive estimation of TL (the detection limit is less than 0.15 kb). This technique is capable of detecting chromosomes with critically short telomeres or discovered chromosomes, an advantage of the technique given the relevance of short telomeres in diseases related to senescence and aging. Besides, this technique can combine TL quantification with karyotype analysis and the study of chromosomal abnormalities (chromosome fusion), and is capable of measuring TL even in a small number of rare cells. Unfortunately, metaphase Q-FISH is not applicable in non-dividing cells and is very difficult in samples with a low proliferation rate. This method is time-consuming, expensive, and labor-intensive, limiting its use in large epidemiological studies. It requires a suitable fluorescence microscope and digital camera equipment, which is expensive and requires a properly trained technician (Lai et al. 2018). Furthermore, there is a limitation in the fluorophores that can be used to label the telomeric probe.

Interphase Q-FISH is performed on interphase cells instead of metaphase chromosomes, which solves the deficiency of Q-FISH in metaphase with respect to its limitation of applicability only in proliferating cells, is less laborious, and requires less time (Montpetit et al. 2014; Lai et al. 2018). Nevertheless, it has the disadvantage that it is not able to measure the size of the telomeres of individual chromosomes or to detect the free ends of telomeres. In contrast, the interphase Q-FISH provides a good estimate of the frequency of short telomeres. An enhancement of interphase Q-FISH is high-throughput Q-FISH (HT Q-FISH), a validated, accurate, and sensitive technology, which evaluates interphase nuclei with high-throughput microscopy in a 384-well format. HT Q-FISH is a precise and sensitive technique capable of measuring TL in almost any type of cell. Its speed and automat (it can process 384 samples with 1000 nuclei per sample in 2 h) makes it suitable for large epidemiological studies, being capable of calculating the frequency of short telomeres and even studying individual telomere spots in a cell. However, it cannot detect telomere free ends and requires confocal microscopy equipment.

The **Flow-FISH methodology** combines the basic concepts of FISH applied to the measurement of TL with the use of cell sorting by flow cytometry, which allows FISH to measure TL in individual cells after they have been classified into different

subpopulations (Rufer et al. 1998; Baerlocher et al. 2006). This technique requires cells in suspension so it is applied mainly in the measurement of telomeres of hematopoietic cells. In a first step, nucleated cells are isolated from the sample and prepared for flow cytometry. The DNA is then denatured (applying heat and formamide under controlled conditions), which allows it to hybridize with a fluorescent probe, preferably a peptide nucleic acid probe, which specifically recognizes telomeric DNA. After the corresponding washes, the DNA is counterstained, using a nonspecific DNA fluorescent dye (4′,6-diamidino-2-phenylindole, DAPI), which allows the quantification of the specific fluorescent signal of the telomeres to be normalized. Finally, the acquisition and flow cytometer analysis takes place. For this, cells are sorted and passed one by one through the lasers and the fluorescent signals, both from the telomeres (specific) and from the rest of the DNA (nonspecific) are measured (Lauzon et al. 2000; Baerlocher et al. 2002). The use of control cells of known TL allows the fluorescent signal to be converted into absolute TL values. The reproducibility and precision of this procedure depends on all the experimental steps and conditions (temperature, reagent concentrations, incubation times ...) being perfectly optimized. This technique requires a low number of cells (less than 100,000) per single, and some of the steps in the procedure can be automated, thus requiring less time compared to Q-FISH in metaphase. These characteristics make Flow FISH an accurate method suitable for medium-scale studies. Flow FISH can be adapted for higher throughput, thus permitting some larger scale studies on human lymphocytes. On the other hand, flow FISH only measures mean TL values per cell and cannot detect individual telomeres or telomere free ends. Cells in the sample need to be fixed, so the chemicals used can affect cell surface epitopes or hybridization efficiency. Furthermore, a laborious technique requires a high level of skill to be performed correctly and a flow sorting equipment (Dweck and Maitra 2021).

In **telomapping**, Q-FISH is performed on biopsies of any type of tissue and thus provides added value in preclinical and clinical studies using formaldehyde-fixed paraffin-embedded archival samples.

STELA Methods

STELA, which was designed to study the short arms of sex chromosomes, measures the abundance of the shortest telomeres using a combination of ligation, PCR-based methods and Southern blot analysis (Montpetit et al. 2014; Baird et al. 2003; Dweck and Maitra 2021). STELA is a highly accurate technique that is able to measure TL ranging from 406 bp to 20 kb. STELA technique is capable to measure the length of telomeres in specific chromosomes and detecting critically short telomeres even with limited starting material (few picograms of DNA or as few as 50 cells), which makes this method a good choice for the study of rare cells and no dividing senescent cells. Although it requires no specialized equipment for telomere analysis, it is a labor intensive and technically challenging technique that requires good optimization of the procedure so it has a low-throughput nature and it is not applicable to large studies.

Not all chromosomal ends have unique sequences, which restricts, a priori, the number of chromosome ends that can be analyzed with this technique. The development of Universal STELA (U-STELA) procedure solved this problem (Bendix et al. 2010). This improved method uses small amounts of DNA and is capable of detecting telomeres at all ends of chromosomes, which makes it possible to monitor changes in the shortest telomere of cells, although it is not effective in detecting TL greater than 8 kb.

U-STELA allows the detection of critical short telomeres in all chromosomes using small amounts of DNA, solving the limitation of conventional STELA. However, it is less useful when measuring mean TL and like conventional STELA is not able to detect telomeres longer than 20 kb.

Single Telomere Absolute-Length Rapid (STAR)

The STAR assay is a novel high-throughput digital real-time PCR approach that measures the absolute lengths and quantities of individual telomeres, in less than 3 h. The STAR assay is an accurate, reproducible, powerful tool that enables the use of TL distribution as a biomarker in disease and population-wide studies (Luo et al. 2020).

Digital PCR platforms allow the random encapsulation of individual molecules, with high throughput and minimal reagent consumption (Fig. 2). After digesting the genomic DNA with restriction enzymes that do not cut within the telomeric sequence, the telomere-specific primers and the qPCR mixture are added, and the mixture is compartmentalized (nanoliters) on the digital PCR chip, with the dilution

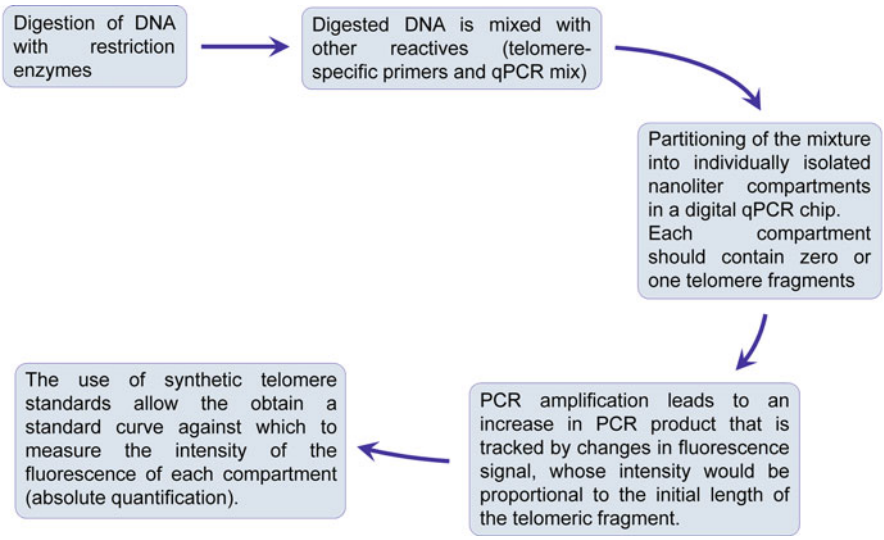

Fig. 2 Representative scheme of the STAR assay procedure

adequate to ensure that each compartment will contain only one telomeric fragment (Dweck and Maitra 2021). With the development of well-characterized standards, this approach allows to measure absolute TL. Thus, combining multiple short, synthetic telomeric sequences (90 bp sequences) that serve as long telomere standards, the STAR assay has a wide dynamic range (0.2–320 kb). Finally, this procedure unbiases comparison across laboratories.

Main Remarks

Numerous methods allow the measurement of TL, although there is no single technique capable of being precise, sensitive, fast, and simple at the same time. Depending on its strengths and limitations, choosing the most appropriate method is critical.

On the one hand, the type of measurement obtained is crucial, since some methods only quantify the mean length of telomeres in a sample (such as TRF and qPCR), of individual cells (FISH flow), or of telomeric stains (interphase Q-FISH). U-STELA and Q-FISH in metaphase can measure the length of individual telomeres of all chromosomes. Given the relevance that the possibility of detecting extremely short telomeres or free ends has acquired in recent years, this critical aspect must be considered when selecting the appropriate method (metaphase Q-FISH, STELA, U-STELA and STAR).

Regarding the type of sample used, all Q-FISH methods require live cells, while TRF, qPCR, STELA, and STAR use DNA directly. Metaphase Q-FISH requires proliferating cells and cannot be performed with senescent cells. On the other hand, and regarding the availability of starting material, TRF is the method that requires the largest amount of sample, while STELA, metaphase Q-FISH, interphase Q-FISH, and STAR require the least amount.

The quantification of telomeres in mice should not be performed with STELA and U-STELA since the upper limit of detection (20 kb) is below the size of murine telomeres. Lastly, TRF, metaphase Q-FISH, and STELA can process a small number of samples, while qPCR, interphase Q-FISH, flow FISH, and STAR are suitable for large-scale studies.

Section 2. Telomere Length: Links with Anthropometry in Lifestyle Intervention

The prevalence of obesity is raised in children and adult's populations worldwide and, due to its impact on the cost of the healthcare system (nearly 21% of all medical spending in the USA), is a number one public health problem. In 2016, globally approximately 39 million children under 5 years of age were obese and 340 million children and adolescents (5–19 years) were overweight or obese (WHO 2021). In Europe 59% of the subjects have either overweight or obesity. Recently, the Europe

Commission recently had classified obesity as a chronic relapsing disease, gateway to other noncommunicable diseases (Burki 2021).

According to the World Health Organization (WHO) obesity is described as an excess fat mass that increases the risk of morbidity, impairment of physical, psychological, or social well-being and/or mortality. It is a multifactorial disease whose etiology involves genetic, metabolic, hormonal, behavioral, environmental, psychological, economic, and social factors (Burki 2021; Marti and Aguilera 2018). It is characterized by an excessive accumulation of energy as fat in the body, leading to an increase in body weight in relation to the expected value according to sex, height, and age. Inflamed or dysfunctional adipose tissue releases inflammatory mediators that trigger low-grade systemic inflammation with an imbalance of oxidative stress that affects the different organs (liver, pancreas, kidney, vascular endothelium, muscle, among others) and increases cardiometabolic risk (Marti and Aguilera 2018). Hence, there is evidence from epidemiological studies that obesity and mortality rate are related, and that as the BMI increases, the risk of suffering from diabetes, cardiovascular disease, cancer, or others diseases also increases.

Inflammation and Oxidative Stress Linked to Obesity and TL

Obesity is associated with both a low-grade inflammation and oxidative stress processes increasing cardiometabolic risk (Marti and Aguilera 2018). Oxidative stress could be defined as the imbalance between production of reactive oxygen species and the antioxidant systems, leading to oxidative damage to cells. Reactive oxygen species are chemical species containing free radicals, namely, hydroxyl radical (OH–), superoxide anion (O_2–), and hydrogen peroxide (H_2O_2). Reactive oxygen species production in adipocytes is mainly caused by the catalytic activity of nicotinamide adenine dinucleotide phosphate oxidase (Marti and Aguilera 2018). Excess adipocytes in obesity lead to increased reactive oxygen species and, as a consequence, increased oxidative stress. Thus, several studies have correlated obesity with oxidative stress (Marti and Aguilera 2018).

Obesity-related inflammation and oxidative stress may modify telomere length (Marti and Zalba 2017). The production of reactive oxygen species could damage all components of the cell, including proteins, lipids, and DNA. The repetitive DNA sequences at telomeres adopt a specific structure called a t-loop structure that is composed of shelterin complexes that protect the DNA. Telomeres are highly sensitive to oxidative stress damage due to their high content of guanines. In fact, short and dysfunctional telomeres are the starting point for cellular senescence, cell death, and DNA instability, being 8-oxo-7,8-dihydroguanine the reactive oxygen species most abundant in senescent cells (Welendorf et al. 2019).

In the literature the relationship between BMI and TL was robustly analyzed in young, middle-aged and older subjects in a meta-analysis with data from 146,114 individuals (87 observational studies). Higher BMI was associated with shorter telomeres, especially in younger subjects (18–60 years old). Notably, telomere loss was −3.99 bp and −7.67 bp per unit increase in BMI in the total sample and in

young adults, respectively (Gielen et al. 2018). A substantial number of observational studies have described associations between modifiable lifestyle behaviors and TL (Arsenis et al. 2017; Marti et al. 2017; Galiè et al. 2020; Canudas et al. 2020; Ojeda-Rodríguez et al. 2021; Alonso-Pedrero et al. 2022), which has led to growing interest in determining whether intervention studies based on lifestyle changes would influence TL.

Lifestyle Factors for Obesity Development

Childhood and adolescence are stages of life in which children acquire and learn healthy or unhealthy lifestyle habits. In this sense, the environment in which these young people live will influence their behavior. As already mentioned, obesity has been described as the result of an imbalance between energy intake and energy expenditure. Today, children have been described as being in an "obesogenic" environment that is characterized by sedentary behaviors and unhealthy diets (Lanigan 2018).

In addition, the family environment can influence children's adiposity through different pathways: (1) the availability of different types of food in the home, (2) the dietary patterns followed by the family, and (3) eating behaviors, where parental attitudes toward children's eating behavior have been shown to have an effect on adiposity levels (Brown and Perrin 2018). Recently, it has been described that unhealthy dietary patterns established during childhood (< 2 years of age) continue into adolescence (Luque et al. 2018).

In relation to lifestyle, in modern society food and drink are more accessible than ever and few children need to engage in a high level of physical activity to get around or choose to do so in their leisure time. Inadequate diet due to excess energy intake, increased consumption of highly palatable foods and sugary drinks, skipping breakfast and snacking between meals are risk behaviors for obesity. Regarding the relationship between physical activity and obesity, high levels of moderate to vigorous PA have been shown to have a protective effect on obesity, while sedentary behavior is a risk factor. Furthermore, the promotion of physical activity in the family context is recommended as a preventive strategy for pediatric obesity (Foster et al. 2018). It should be noted that children's and young people's leisure time is increasingly physically inactive, with time spent watching television and playing video games (sedentary activity) playing a major role.

Obesity has increased over the last 50 years, reaching high levels in many countries. It is considered the number one health problem of the twenty-first century and multiple approaches have been proposed to reduce the number of obese individuals. As obesity in childhood continues into adulthood, treatment and prevention in the youth population is a key factor in reducing its incidence.

Intensive lifestyle programs have been shown to be the best approach to reduce excess weight. Obesity is characterized by excess energy intake and low energy expenditure. For this reason, strategies aimed at modifying these aspects should be considered. They consist of a combination of three areas: dietary treatment,

promotion of physical activity, and behavioral therapy. Therefore, a multidisciplinary team composed of physicians, dietitians, psychologists, nurses, and physical activity experts is needed to conduct lifestyle interventions. They are the gold standard for reducing cardiometabolic risk through lifestyle change and weight loss.

Dietary Treatment

Dietary recommendations for obesity include nutritional counseling, reducing refined carbohydrates, baking, meat and saturated fat intake, and increasing consumption of fruits, vegetables, whole grains, fish, and legumes. There are different dietary patterns that have been shown to be effective for weight loss and improvement of metabolic comorbidities. These patterns are known under the following names: Optimized Mixed Diet, the Mediterranean Diet (MD), the New Nordic Diet, and the dietary approach to stop hypertension, also known as the DASH dietary pattern.

The Mediterranean Diet is a healthy dietary pattern typical of countries bordering the Mediterranean Sea such as Greece, Italy, Spain, and others. High adherence to the DM has been found to be associated with lower increases in BMI (Tognon et al. 2014). It is characterized by a high consumption of vegetables (2 servings per day), fruits (3 servings per day), whole grains, legumes, nuts and olive oil, a moderate consumption of poultry, fish, and dairy products; and a lower consumption of processed meats. This pattern is accompanied by different lifestyle factors, such as the way food is cooked, the use of spices and herbs instead of salt, physical activity or the enjoyment of food with family or friends. Obese children following a lifestyle intervention with DM have been shown to decrease BMI-SDS and some components of metabolic syndrome such as triglycerides, HDL-cholesterol, and glucose levels. The presence of metabolic syndrome was reduced in 45% of participants (Velázquez-López et al. 2014). Furthermore, in the PREDIMED study, following a Mediterranean-type dietary pattern in adult subjects at high cardiovascular risk has been shown to be a prevention strategy in cardiovascular disease endpoints (Estruch et al. 2018; García-Calzón et al. 2017).

Promoting Physical Activity

As mentioned above, obesity is the result of an imbalance between energy intake and energy expenditure, with physical activity being a key point in the treatment of obesity. The main problems of obese young people are: (1) lack of physical activity (PA) and (2) high levels of sedentary activity.

The WHO advises that children and adolescents should spend more than 60 min per day in moderate to vigorous physical activity that leads to an increase in heart rate of at least 65–70%. This recommendation was based on evidence of favorable associations between PA and adiposity or cardiometabolic biomarkers. Several studies report that European children do not reach this recommendation, with the average time spent in moderate to vigorous PA being 36 min/day (Konstabel et al. 2014). Neither obese nor overweight European children and adolescents follow the PA recommendations (Hughes et al. 2006). A recent meta-analysis has shown that obese children are less active than those of normal weight (Poitras et al. 2016).

On the other hand, lifestyle interventions based on dietary changes and promotion of physical activity or behavioral therapy have shown to be more effective than dietary interventions alone (Henriksson et al. 2018).

Notably, sedentary lifestyles have been considered one of the main risk factors for obesity. In this line, several global organizations have expressed the recommendation of not spending more than 2 h a day on screen activities such as: watching TV, playing video games, and spending time with tablets or smartphones. Screen time is used to extrapolate sedentary behavior because it accounts for the majority of sedentary time in children and is associated with adverse health effects (Henriksson et al. 2018).

Furthermore, it is important that lifestyle interventions do not focus physical activity recommendations on a single behavior (PA, sedentary or sleep time), but take into account all 24 h of the day. A recent review found that children and adolescents with lower levels of adiposity were those with a combination of optimal physical activity and behavior (i.e., high PA, low sedentary time, and sufficient sleep) (Chaput et al. 2017).

Behavioral Therapy

One of the main goals of lifestyle intervention is to achieve an improvement in anthropometric and metabolic outcomes, and to maintain the habits that have led to this improvement (van Hoek et al. 2016). In this context, behavioral therapy during lifestyle intervention is a key element to achieve improvement in eating behaviors and PA. This therapy encompasses various actions including counseling on self-monitoring of PA and eating behaviors, stimulus control, action planning, goal setting, and other behavior modification strategies (van Hoek et al. 2016).

Given that children and adolescents live with their families and that parents exert an important influence on healthy behaviors, it is important that behavioral interventions include the family. Lifestyle interventions that take the family into account have been described as more successful than those that only focus on children (Wilfley et al. 2017). The increasing rates of childhood and adolescent obesity appear to be the result of an interaction between genetic predisposition with lifestyle factors such as excess caloric intake and reduced physical activity. With this "obesogenic" environment, it is difficult to maintain a micro-environment that protects children and adolescents from obesity. The physical activity levels of parents or guardians, their eating habits, television viewing, and other sedentary attitudes play a special role. Therefore, intervention program based on lifestyle changes are more effective when the family is involved.

Lifestyle Intervention Studies

Most studies that have evaluated TL and lifestyle factors are cross-sectional analyses, which have not been able to consider cause and effect (García-Calzón and Marti 2017). But, intervention studies are a very important tool in epidemiology. The fundamental difference with cohort studies is that in intervention studies, the exposure is decided and allocated by the researcher and its effect is evaluated. These

studies represent the last link in the progression of epidemiological reasoning, because if properly designed and executed, they provide the strong and direct epidemiological evidence to judge the existence of a causal relationship between an exposure and an effect.

A recent review indicated that interventions involving multiple lifestyle modification components (i.e., diet and exercise) aimed to weight loss may influence TL (Qiao et al. 2020). They showed that six of the seven selected studies reported a significant intervention impacts in delaying telomere shortening suggesting that weight loss could reversed accelerated telomere shortening. It is crucial to design lifestyle intervention (based on diet, PA, or both types of recommendations) to be effective for maintenance of telomere length and also to determine to what extent weight loss is central.

Here we summarized human studies assessing the effect of lifestyle intervention aimed to reduce cardiometabolic risk on TL in adult and pediatric populations in Table 2 (Ojeda-Rodríguez et al. 2018a, b, 2020). For all of them TL was measured in blood cells by qPCR. Studies measuring TL should pay meticulous attention to the protocol and method used to measure TL. In line with this, in our studies we considered several points to reduce potential measurement error: TL was measured by MMqPCR, in which the quantification of telomeres and the single copy gene was performed in a single reaction and DNA samples corresponding to different time points (e.g., 0, 2, and 12 months) were run by triplicate in the same plate. Briefly, there are three lifestyle intervention studies that we performed, one in adult subjects at high cardiovascular risk (the PREDIMED-Navarra) and two in pediatric populations with abdominal obesity (the EVASYON project, and IGENOI study):

- The PREDIMED is a large randomized clinical trial of dietary intervention in subjects at high cardiovascular risk with the main objective of finding out whether the Mediterranean diet supplemented with extra virgin olive oil or nuts prevents the onset of cardiovascular disease compared to a low-fat diet (Estruch et al. 2018). (More information is available ISRCTN 35739639 and at http://www.predimed.org/.)
- The EVASYON project consists of the development, implementation, and evaluation of the effectiveness of a therapeutic program for overweight and obese adolescents: comprehensive nutrition and physical activity education (Martinez-Gomez et al. 2009). It is a national pilot intervention program aimed at establishing a useful educational program specifically targeted at overweight and obese adolescents (http://www.ame-ab.es/cms/alimentacion-y-salud/proyectos/proyecto-evasyon/).
- The IGENOI study is a two-year family-based lifestyle intervention study involving children and adolescents with abdominal obesity. It is a randomized controlled clinical trial (NCT03147261) in which the control group received standard pediatric recommendations on healthy diet, while the intensive care group was advised to follow a moderately hypocaloric Mediterranean diet. Both groups were encouraged to accumulate an extra time of 200 min of physical activity per week at 60–75% of their maximum heart rate (Ojeda-Rodriguez et al. 2018a, b).

Table 2 Human studies assessing the effect of lifestyle intervention aimed to reduce cardiometabolic risk on TL in adult and pediatric populations

Main findings	Type of study	Sample characteristics	Publication
Adult subjects			
Baseline TL is inversely associated with changes in obesity parameters after the intervention. A reduction in obesity indices when an increase in TL is observed after 5 year of MD intervention	Nutritional program to follow the Mediterranean diet for 5-year (all subjects together)	520 subjects with high cardiovascular risk from the PREDIMED-Navarra study	García-Calzón et al. (2014a)
A greater baseline adherence to MD was linked to longer telomeres in women. No beneficial effect of following an MD compared to low-fat diet in telomere erosion	Nutritional program to follow the Mediterranean diet for 5-year supplemental foods (olive oil or mixed nuts), control group were advice to follow a low-fat diet	520 subjects at high cardiovascular risk from the PREDIMED-Navarra study	García-Calzón et al. (2016)
Increase in the TL over the 4.5 year period both in the intervention and in the control group	Lifestyle intervention (4.5 years of follow-up)	311 adults with impaired glucose tolerance	Hovatta et al. (2012)
Changes in TL were associated to baseline TL. No changes in telomere length in any intervention groups compared to the control group.	12-Month program with a 10% weight loss goal, or an increase in moderate to vigorous exercise, or both, plus Control group (no intervention)	439 postmenopausal overweight or obese women	Mason et al. (2013)
No changes in telomere length in any intervention groups compared to the control group. Weight-loss maintenance of 10% or more was associated with larger telomeres 12 month later	5,5-Month weight loss program (and follow up to month 12) with or without mindfulness training with identical diet-exercise recommendations	194 adult with abdominal obesity	Mason et al. (2018)
Changes in TL were associated to baseline TL. No change in telomere length between intervention groups was observed	6-Month weight loss program with or without exercise prescription	50 adult with abdominal obesity	Svenson et al. (2011)
Weight-loss after a bariatric surgery intervention was associated with larger telomeres	Lifestyle education 6-Month program after bioenteric intragastric balloon	42 obese subjects	Carulli et al. (2016)

(continued)

Table 2 (continued)

Main findings	Type of study	Sample characteristics	Publication
Children and adolescents			
Weight loss intervention is accompanied by an increase in LTL. Initial longer LTL predicts a better weight loss response	Lifestyle Intervention based on dietary and PA recommendations (EVASYON): 2 month of an energy restricted diet and 6 months of follow up	74 adolescents aged 12–16 years with obesity	García-Calzón et al. (2014b)
Longer telomeres at baseline were associated with a higher reduction in glucose and IL-6 serum levels after 2 months of the weight-loss treatment	Lifestyle Intervention based on dietary and PA recommendations (EVASYON): 2 month of an energy restricted diet and 6 months of follow up	66 children aged 12–16 years with abdominal obesity	Garcia-Calzón et al. (2017)
Inverse correlation between TL and obesity traits was observed in children with abdominal obesity. Baseline TL could predict changes in blood glucose levels	Lifestyle Intervention based on dietary and PA recommendations (IGENOI): 2 month of an energy restricted diet and 10 months of follow up	106 children aged 7–16 years with abdominal obesity	Morell-Azanza et al. (2020)
Favorable changes in diet quality indices could contribute to telomere integrity	Lifestyle Intervention based on dietary and PA recommendations (IGENOI): 2 month of an energy restricted diet and 10 months of follow up	87 children aged 7–16 years with abdominal obesity	Ojeda-Rodríguez et al. (2020)
Changes in physical activity had a direct effect on TL	Lifestyle Intervention based on dietary and PA recommendations (IGENOI): 2 month of an energy restricted diet and 10 months of follow up	121 children aged 7–16 years with abdominal obesity	Ojeda-Rodríguez et al. (2021)

Interestingly, we reported that TL is linked to anthropometry changes in adult and adolescents subjects. In the PREDIMED-Navarra study, TL was measured at baseline and after 5 years follow-up in subjects at high cardiovascular risk (García-Calzón et al. 2014a). We ran a multiple regression model to predict changes in adiposity indices at year 5 according to baseline LT. We found that higher LT at baseline significantly predicted greater reductions in body weight, body mass index, and waist circumference adjusted for age, sex, and the corresponding anthropometric variable at baseline. Moreover, an association was also found between changes in LT during the intervention period and anthropometric variables after 5 years of the nutritional intervention based on the MD pattern. The reduction in markers of adiposity, associated with increased LT, was even greater in those with longer telomeres at baseline. Interestingly, the *odds ratio* of remaining obese after

5 years, for those with the longest TL, was 0.27 among those who increased TL, and 0.43 among those who decreased TL during follow-up. In regard to this, a trend was observed: the higher the LT at baseline and the greater the increase in LT, the lower the risk of remaining obese. In the EVASYON study, TL was measured in 74 overweight or obese adolescents at baseline and after 2 or 6 months of follow-up. They significantly lost body weight and had a significant improvement in cardiometabolic parameters (García-Calzón et al. 2014b, 2017). The multiple regression models allow us to predict changes in anthropometric index according to LT at baseline. In adolescent, during the 2-month intensive treatment phase, LT at baseline significantly predicted a greater reduction in body weight and BMI-SDS. Interestingly, males with higher baseline LT also had a greater decrease in body weight and BMI-SDS after 6 months of the multidisciplinary intervention.

It is also noticeable that weight loss in some studies was associated to longer TL (Mason et al. 2018; Carulli et al. 2016; Hovatta et al. 2012; García-Calzón et al. 2014b). Similarly, our group also found that TL was associated with an improvement in glucose levels in lifestyle intervention studies (García-Calzón et al. 2017; Morell-Azanza et al. 2020). On the other hand, several intervention studies indicated that changes in TL are associated to baseline TL (Mason et al. 2013; García-Calzón et al. 2014b).

Furthermore, the association between TL and changes in lifestyle factors in intervention programs aimed to lower cardiometabolic risk was evaluated in several research works. No evidence for an improvement on TL was reported when a healthy lifestyle was compared to that of the control group (Mason et al. 2013, 2018; Hovatta et al. 2012; Svenson et al. 2011, García-Calzón et al. 2016). But, in women of the PREDIMED-Navarra the adherence to Mediterranean diet (14 item questionnaire) was found to be linked to longer telomeres at baseline (García-Calzón et al. 2016). Moreover, in a pediatric population with abdominal obesity favorable changes in diet quality or PA seem to influence telomere integrity in the IGENOI study. Notably, each increase in 1 MET (metabolic equivalent) in the PA level was independently associated with a 26-units increase in TL, measured as the ratio T/S. Also, we observed that the model that account for the METs explained up to 58% of the variability in TL (Ojeda-Rodríguez et al. 2021). It is known that regular physical activity has also been associated with decreased levels of oxidative stress and inflammation, which affect telomere shortening (Himbert et al. 2018).

In spite of the differences in the study design and methodology, limitations, and pitfalls of the studies, it seem that programs to prevent excess weight or associated comorbidities may also contribute to ameliorate telomere attrition. However, more interventions are needed to confirm the benefits of lifestyle changes on telomere dynamics (Erusalimsky 2020). Furthermore, it is worthy to mention that that genetic variant may help to explain different responses to lifestyle interventions. For example, García-Galzón et al. (2015) reported that Ala carrier subjects for the PPARG2 gene (rs1801282) with a high adherence to the MD pattern exhibited increased TL after 5 year follow-up. In addition, effective intervention strategies to delay telomere shortening should examine the complex interaction between environmental factors including health habits and behaviors (lifestyle), and genetic, epigenetic, and other

"omics" markers. The new challenge is to integrate "multi-omics" data together with lifestyle data and to discover new markers (telomere length) to successfully deliver and evaluate individualized interventions as required for Precision Nutrition (Medicine).

TL as a Biomarker in Lifestyle Intervention

Substantial weight loss is able to lower chronic inflammation and adipose tissue oxidative stress and can lead to promote TL conservation and DNA repair, thus reduction telomere attrition. One important finding coming from the intervention studies compiled here is the association between TL and adiposity changes in lifestyle interventions, suggesting that TL may be used as a **biomarker of response** (Welendorf et al. 2019). Thus, this blood biomarker may serve as a noninvasive, low cost, and time-efficient tool to assess effectiveness of lifestyle programs for lowering cardiometabolic risk.

Furthermore positive effects on TL could be also related with a reduction in saturated fat and sugar consumed, as well as with an increase in vitamin and mineral (antioxidant) intake. TL may not only reflect one nutrient but may be associated with a dietary pattern or food group. In this regard, we found a higher risk of short telomeres linked to an inflammatory diet (in the PREDIMED-Navarra study, Am J Clin Nutr. 2015;102(4):897–904) or high consumption of ultraprocessed food (Am J Clin Nutr. 2020;111(6):1259–1266, Alonso-Pedrero et al., 2020) while a lower risk of shorter telomeres was associated with a high adherence to MD in cross-sectional studies with older subjects from the SUN study (Clin Nutr. 2020;39(8):2487–2494, Ojeda-Rodriguez et al. 2020). Findings from our work and others suggest that the MD pattern helps to lower inflammation and oxidative stress and could provide protection for telomeres.

In conclusion, there is a need for the identification of dynamic biomarkers that may predict weight loss and could help in obesity management for the prescription of most suitable – individual – lifestyle changes. However, inconsistent results concerning the benefit of lifestyle intervention on TL suggest the need for more studies – probably devoted to the measurement of ***excessively short telomeres*** – before its clinical application in routine use as biomarker.

Applications to Prognosis or Applications to Other Diseases or Conditions

Applications to Prognosis

In this chapter an association between TL and measures of adiposity is described in adult and adolescent subjects enrolled in lifestyle interventions (García-Calzón et al. 2014a, b). Also, a recent review indicated that interventions involving lifestyle modification components (i.e., diet and exercise) aimed to weight loss may influence TL (Qiao et al. 2020). One important finding coming

from the evidence compiled here is the association between TL and measures of adiposity in lifestyle interventions, suggesting that TL may be used as a ***biomarker of response*** *in adults and adolescent subjects (Welendorf et al. 2019). Thus, this blood biomarker may serve as a noninvasive, low cost marker to assess effectiveness of lifestyle programs aimed to lower cardiometabolic risk.*

Applications of *short telomeres* measurement as a new clinical marker

In this chapter, it is stated that abnormal telomere shortening underlies patients' risk of developing age-related degenerative diseases, including metabolic (diabetes, obesity, etc.) and cardiovascular diseases, and several cancers. Although it is indisputable that telomere shortening is a characteristic associated with these pathologies, the simple quantification of the mean telomere size of a sample is an insufficient surrogate marker, and it is necessary to develop new tools in biomedical research and clinical practice, capable of measuring telomeres and specifically identify those ***excessively short telomeres****, as the true characteristics of diseases related to aging (Vera and Blasco 2012).*

Mini-Dictionary of Terms

- **Canonical sequence:** DNA sequence that reflects the most common choice of bases at each position. In the case of telomeres, this sequence is repeated thousands of times.
- **Cardiometabolic risk:** It describes a person's chances of having a cardiovascular event such as heart attack or stroke when one or more risk factors are present. Some major risk factors include: obesity, high LDL ("bad") cholesterol, high blood fat (triglycerides), low HDL ("good") cholesterol, high blood pressure, diabetes, smoking, and tobacco.
- **-Clinical trial**: It is a formal study carried out according to a prospectively defined protocol that is intended to discover or verify the safety and effectiveness of procedures or interventions in humans (US National Library of Medicine 2019).
- **8-oxo-7,8-dihydroguanine**: The reactive oxygen species most abundant in senescent cells.
- **Fluorescence in situ hybridization (FISH)**: It is a laboratory technique for detecting and locating a specific DNA sequence on a chromosome. A fluorescently labeled DNA probe is capable of specifically detecting its complementary DNA sequence present on the chromosomes of a cell.
- **High-throughput technologies:** Those in which exist automation of experiments such that large-scale repetition becomes feasible. In the case of the molecular biology studies, those kinds of technologies allow sequencing of DNA and RNA, amplification of DNA fragments by PCR, etc., in a rapid and cost-effective manner.
- **Intervention:** It refers to any drug, device, biologic, behavioral modification, nutritional modification, lifestyle modification, or other treatment intended to improve health.

- **Inflammation:** A local response to cellular injury that is marked by capillary dilatation, leukocytic infiltration, redness, heat, pain, swelling, or loss of function and that serves as a mechanism initiating the elimination of elimination of foreign substances and for healing damaged tissue.
- **Mediterranean diet:** It is a generic term based on the traditional eating habits in the 16 countries bordering the Mediterranean Sea. A Mediterranean-style diet typically includes: plenty of fruits, vegetables, bread and other grains, potatoes, beans, nuts, and seeds; olive oil as a primary fat source; and dairy products, eggs, fish, and poultry in low to moderate amount.
- **MET**: One metabolic equivalent is defined as the amount of oxygen consumed while sitting at rest and is equal to 3.5 ml O_2 per kg body weight x min.
- **Oxidative stress:** It reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of reactive oxygen species (peroxides and free radicals) that damage all components of the cell, including proteins, lipids, and DNA.
- **Randomized controlled trial:** A study in which the participants are assigned by chance to separate groups that compare different treatments; neither the researchers nor the participants can choose which group. Using chance to assign people to groups means that the groups will be similar and that the treatments they receive can be compared objectively. At the time of the trial, it is not known which treatment is best.
- **Telomeres:** Regions of repeating noncoding nucleotide sequences at the end of each chromosome. Their main function is to protect the ends of the chromosomes from degradation and maintain the integrity of the chromosomes.

Key Facts of TL Measurement

Some techniques used to measure telomeres may include non-canonical sequences from the subtelomeric region, which may overestimate the reliability of the measurement.

Short telomeres are a characteristic of diseases related to senescence and aging, so the inability to detect them represents a significant lack of information

Key Facts of Obesity

Obesity prevalence is rising worldwide being a number 1 problem in public health.

Obesity is accompanied by inflammatory and oxidative stress processes that partly explain the increased cardiometabolic risk and the comorbidities associated with obesity.

Lifestyle changes are useful strategies for obesity prevention and treatment.

Key Facts of Lifestyle Intervention

Lifestyle interventions aimed to lower cardiometabolic risk encourage participants to follow healthy dietary and physical activity recommendations.

There are many types of interventions; an important issue is the number and characteristics of subjects, experimental design, and duration of the follow-up.

Interventions that are able to increase the adherence to Mediterranean diet are successful in reducing cardiometabolic risk.

Summary Points

- The detection of excessively short telomeres emerges as an important new tool in biomedical research and clinical practice.
- Successful lifestyle intervention (those able to reduce cardiometabolic risk) may ameliorate inflammatory and oxidative stress processes ameliorating telomere erosion.
- Research works indicate that telomere length varies by age and sex, but there are limited data to evaluate the clinical relevance of these differences.
- Lifestyle factors that impact telomere length include exercise, smoking, measures of adiposity, alcohol, among others.
- Some studies showed that changes in telomere length are associated with changes in adiposity measures in adolescents and adults after lifestyle interventions.
- Changes in physical activity levels had a direct effect on telomere length, a biomarker of cellular aging and oxidative stress.

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GWAS and GWAIS for Identifying Connections Between Genetics, Nutrition, and Health: The Example of Omega-3 and Plasma Triglycerides

27

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Abstract

Given the large interindividual variability observed in the plasma lipid responsiveness to nutritional intake, researchers are interested in deciphering this heterogeneity using a precision nutrition framework based on omics technologies. Genetic variability is one factor contributing to the observed interindividual variability. To study this, researchers are using genome-wide association studies (GWAS) and its related version for interactions, genome-wide association and interaction studies (GWAIS). These studies may help to identify genetic markers

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contributing to plasma lipid responsiveness to nutrients, foods or dietary patterns. In this chapter, we explore the GWAS and GWAIS approaches further, while focusing on the example of omega-3 fatty acids and their impact on plasma triglyceride levels. When applied to nutrition research, this approach has led to the development of a genetic risk score that can be used to help design precision nutrition applications related to omega-3 fatty acids (EPA + DHA) and resulting changes in plasma triglycerides levels.

Keywords

Omega-3 · GWAS · GWAIS · Genetics · Nutrigenetics · Gene-diet interactions · Nutrition · Precision nutrition

Abbreviations

ALA	Alpha-linolenic acid
ComparED	Comparing EPA to DHA
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAS	Fatty Acid Sensor
GWAIS	Genome-wide association and interaction study
GWAS	Genome-wide association study
NIH	National Institutes of Health
SNP	Single nucleotide polymorphism
TG	Triglycerides

Introduction

Precision nutrition aims to optimize health-related responses to dietary components through more tailored dietary approaches (NIH 2020). The National Institutes of Health (NIH) has deemed this area of nutrition a key component of the 2020–2030 strategic direction (NIH 2020). One way to better tailor nutrition recommendations to the individual level is to consider how genetic variability influences responses to nutrition. This science is referred to using the umbrella term *nutritional genomics*. Most studies in nutritional genomics seek to identify associations between a single genetic variant, a dietary component and a health outcome (see, for example, Roke and Mutch 2014). More recently, nutritional genomics researchers have begun to conduct genome-wide association studies (GWAS) as well as genome-wide association and interaction studies (GWAIS), which essentially are studies that test several genetic variations and determine if there are links between these genetic variations and health responses to nutrition. GWAS and GWAIS aim to identify multiple genetic variations contributing to the interindividual variability observed in health outcomes in responses to a dietary component. For example, Rudkowska et al. (2014) used a GWAS approach to develop a nutrigenetic risk score (nutri-GRS) that explained over 20% of the variability in triglyceride (TG) response to an omega-3 fatty acid supplementation (Rudkowska

et al. 2014). Vallée Marcotte et al. later built on this work and developed a more refined nutri-GRS that explained 50% of the variability in the TG response to omega-3 fatty acids (Vallée Marcotte et al. 2019). This chapter will provide an overview of the use of GWAS and GWAIS in nutritional genomics, while focusing on omega-3 fatty acids and their impact on plasma TG levels as a key example.

Overview of Precision Nutrition

The National Institutes of Health (NIH) defines precision nutrition as a "holistic approach to developing comprehensive and dynamic nutritional recommendations relevant to both individual and population health" (NIH 2020). It is a "framework canvassing a wide array of simultaneous influences including genetics, dietary habits and eating patterns, circadian rhythms, health status, socioeconomic and psychological characteristics, food environments, physical activity, and the microbiome" (NIH 2020) (Fig. 1). Precision nutrition recognizes that a one-size-fits-all approach to dietary recommendations is not optimal (Stover and King 2020). Research shows that there is a large interindividual variability in health-related responses to the same

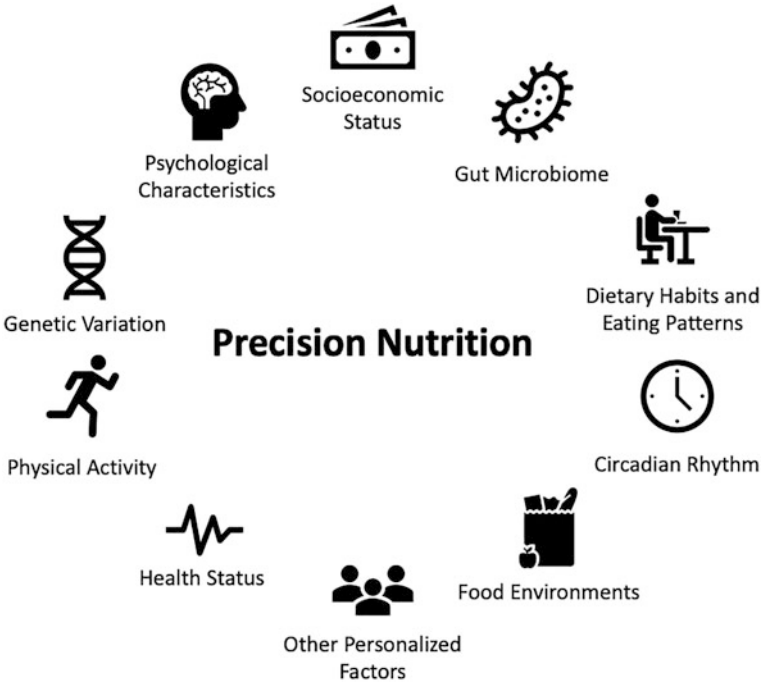

Fig. 1 The precision nutrition framework. The science of precision nutrition considers multiple interactions between genetic variation, psychological characteristics, socioeconomic status, the gut microbiome, dietary habits and eating patterns, food environments, circadian rhythms, physical activity, health status, etc. (NIH 2020)

nutrition intervention; this can be explained partly by differences in the genetic background of individuals (Vallée Marcotte et al. 2019). Individual genetic variation can influence nutrient metabolism and absorption, taste perception and preferences, coagulation, and many other physiological processes (Hughes et al. 2018; Diószegi et al. 2019). As such, genetic variation influences both disease risk, as well as nutritional needs. It should be noted that genetics is just one component of precision nutrition. Precision nutrition also recognizes that the timing of meals, snacks and beverages, reasons for eating/drinking, and how we consume our food and beverages may be just as important as what we eat (NIH 2020).

The Interplay Between Nutrition and Genetics

Given that differences in genetic variation can influence various physiological processes, including nutrient metabolism and absorption, there is an important interplay between nutrition and genetics. There are generally three terms used to describe the connection between nutrition and genetics: *nutrigenomics, nutrigenetics*, and *nutritional genomics*. According to the International Society of Nutrigenetics/Nutrigenomics, *nutrigenomics* generally refers to the influence of nutritional intervention/exposures on gene expression, which in turn affects the proteome and the metabolome (Ferguson et al. 2016). *Nutrigenetics* generally refers to the influence of genotype on health responses to diet (Ferguson et al. 2016). *Nutritional genomics* is typically the umbrella term used to describe both nutrigenetics and nutrigenomics (Sales et al. 2014; Ferguson et al. 2016; Morrison et al. 2020). However, it should be noted that these three terms are often used interchangeably, with their precise definitions debated in the field (Horne et al. 2021).

Most nutrigenetic research focuses on identifying single nucleotide polymorphisms (SNPs) in a single gene (one that is hypothesized to be associated with an outcome) influencing health responses to nutrition. A SNP is the most common type of human genetic variation, whereby one single nucleotide is substituted with another (Fig. 2) (Shastry 2002). Indeed, SNPs in a single gene may impact nutritional needs and health responses to certain nutritional components. For example, minor allele homozygotes for *FADS1/2* SNPs (rs174545, ra174583, rs174561, or rs174537; all in strong linkage disequilibrium with each other according to "SNiPA – a Single Nucleotide Polymorphisms Annotator and Browser" n.d.) exhibit enhanced conversion of alpha-linolenic acid (ALA) into eicosapentanoic acid (EPA), and therefore ALA intake may be more cardioprotective in minor allele homozygotes of these *FADS1/2* SNPs (Gillingham et al. 2013), though more research is needed to investigate these potential cardioprotective effects. While nutrigenetic

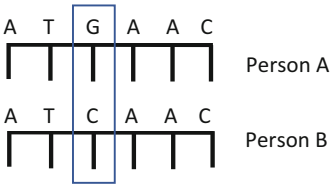

Fig. 2 Single nucleotide polymorphism (SNP). This figure depicts an SNP, where one nucleotide is substituted for another

testing and associated personalized dietary advice based on SNPs in a single gene and even on a single SNP can be highly valuable, it is perhaps more accurate to identify and test for multiple SNPs in multiple genes to develop personalized, genetic-based dietary approaches.

What's a GWAS?

The GWAS approach is frequently used to identify multiple genetic factors that contribute to complex diseases (Amos 2007). In a GWAS, researchers test a number of genetic variants and test for associations between genetic variations, in multiple SNPs, and their association with outcomes of interest. For a nutrigenetic GWAS, outcomes are typically focused on responses to nutrition, such as the change in plasma TG levels following an omega-3 fatty acid supplementation (Vallée Marcotte et al. 2016). In medical genetics, a GWAS seeks to determine the genetic factors contributing to disease development, which can in turn be fundamental for understanding the biological mechanisms driving these diseases (Amos 2007). In nutrigenetics however, a GWAS (or GWAIS – further discussed below) includes an additional layer (nutrition) to explore how nutritional intake may mitigate health or disease outcomes, depending on genetic variation. For a nutritional genomics GWAS, researchers seek to identify how responses to a nutrition intervention are linked to genetic variation. Study participants complete a nutrition intervention, and an outcome of interest is assessed. Participants are then classified as responders, nonresponders, or adverse responders to the nutrition intervention. Connections between these groupings and variations in genetic profiles (i.e., differences in allele frequencies) are then assessed.

Various sequencing technologies can be used in a GWAS for genotyping participants. Collectively referred to as high-throughput sequencing technologies, these approaches allow for the relatively quick analysis of multiple genetic variants at a time – up to hundreds of millions of variants (Churko et al. 2013). In whole genome sequencing, the entire genome (or close to the entire genome) is analyzed in a single process (Centers for Disease Control and Prevention 2019). Whole exome sequencing is another method of genotyping, though less comprehensive than whole genome sequencing. It involves sequencing the entire exome, which is the portion of genetic material that is transcribed into mRNA, or exons (Churko et al. 2013). Studies may also prioritize certain genes for genotyping analysis based on the biological plausibility that they can contribute to the outcomes of interest [see for example: (Hamza et al. 2011) and (Vallée Marcotte et al. 2016)].

What's a GWAIS?

For gene-lifestyle interactions, such as gene-diet or nutrigenetic interactions, the term GWAIS (genome-wide association and interaction study) is emerging for describing observational studies that aim to identify genetic factors contributing to disease, but where lifestyle factors contribute to increasing or decreasing a genetic risk. For example, one GWAIS assessed the interaction between genetic variation,

coffee intake, and risk of developing Parkinson's disease and found that SNPs in *GRIN2A* (a gene which plays a role in regulating excitatory neurotransmission in the brain) were associated with risk of Parkinson's disease, dependent on caffeine intake (Hamza et al. 2011). Individuals with the CC genotype (*GRIN2A* rs4998386) who were heavy caffeine drinkers had an 18% lower risk of developing Parkinson's disease compared to those with the same genotype who were light caffeine drinkers. Moreover, individuals with the TC genotype (*GRIN2A* rs4998386) who were heavy caffeine drinkers had a 59% lower risk of Parkinson's disease, and individuals with the TT genotype had an 81% lower risk compared to the CC genotype; in light caffeine drinkers, genotype did not increase or decrease the disease risk (Hamza et al. 2011).

Overall, both the GWAS and the GWAIS approaches can be used in nutrigenetic research. The GWAS approach analyzes the prevalence of genetic variations and an outcome related to the response to nutrition, which is typically evaluated through an interventional study [e.g., change in TGs in response to an omega-3 fatty acid supplement intervention: (Vallée Marcotte et al. 2016)]. In the GWAIS approach, a gene-diet interaction term is included in the statistical model in order to evaluate associations between genetic variation, dietary intake, and health outcomes, which are typically measured through an observational study [e.g., associations between genetic variation, caffeine intake, and risk of Parkinson's disease (Hamza et al. 2011)].

Example: Using the GWAS Approach for Determining Gene-Diet Associations Related to TG and Omega-3 Fatty Acids

The following provides an overview of how the GWAS approach has been used for identifying associations related to genetics, nutrition, and health, specifically looking at research related to the impact of omega-3 fatty acids on plasma TG responses.

Variability in Plasma TG Response to Omega-3 Fatty Acids

While GWAS and GWAIS have focused on several associations between genetic variations, dietary factors, and health/disease outcomes, many of the findings from this research require further studies and replication; our knowledge of these interactions at this point is only in its infancy. Perhaps the area of research using a GWAS or GWAIS approach that is the most well understood relates to omega-3 fatty acids and plasma TG levels. As such, this will be the focus of the example provided in this chapter.

Recommending EPA + DHA for TG reduction is a common practice among healthcare professionals given that elevated TG is generally linked to worsened cardiometabolic health. Indeed, there is high-quality evidence to support this recommendation (Abdelhamid et al. 2018). However, while population-based research generally demonstrates a clear association between higher dietary or supplemental

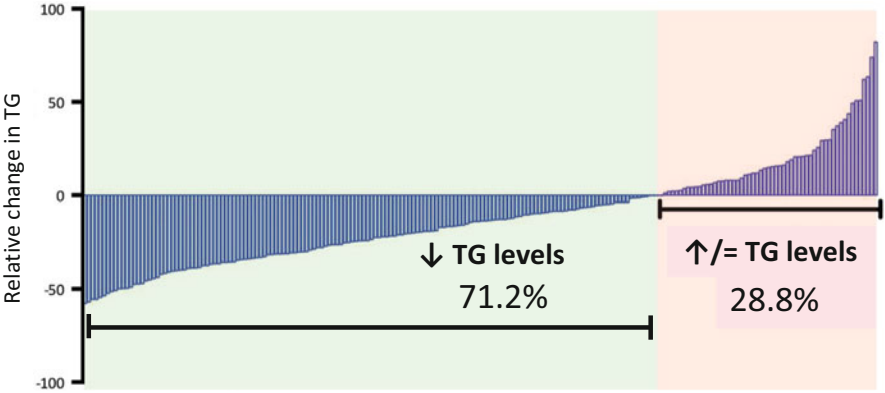

Fig. 3 Example of the interindividual variability in the plasma TG response to EPA + DHA supplementation. (Unpublished data from Vohl, M-C et al.)

omega-3 fatty acid intake and lower plasma TG levels (Abdelhamid et al. 2018), there is substantial interindividual variability in responses to omega-3 fatty acid intake on TG levels. Through interventional research in the Fatty Acid Sensor (FAS) study at Dr. Vohl's lab, Rudkowska et al. recognized this large interindividual variability in the TG lowering response to omega-3 fatty acids (Rudkowska et al. 2014), as exemplified in Fig. 3. This group first conducted an intervention whereby participants consumed approximately 3 g/day of omega-3 fatty acids in the form of EPA + DHA and plasma TG responses to the EPA + DHA supplement were assessed. Participants were then stratified into two groups: responders and non-responders. Responders were considered those who exhibited reduced TG levels following the EPA + DHA supplementation, whereas nonresponders were considered those who exhibited no changes, or increases in TG following the EPA + DHA supplementation (Rudkowska et al. 2014).

A GWAS approach has been used to assess whether there were significant differences in the frequency of SNPs between responders and nonresponders. The results showed that there were 13 SNPs demonstrating suggestive associations with the TG responsiveness to omega-3 fatty acid (EPA + DHA) supplementation. Three of these SNPs were in strong linkage disequilibrium (i.e., they could be used as surrogate markers of each other). As such, ten individual SNPs were suggestively associated with the TG response to EPA + DHA. From there, a nutrigenetic risk score (nutri-GRS) was developed as a tool that can be used to predict if someone would be a responder or a nonresponder to EPA + DHA for plasma TG lowering. SNPs associated with TG responsiveness are scored using the nutri-GRS; individuals with lower scores are more likely to be responders, whereas individuals with higher scores are more likely to be nonresponders to EPA + DHA for TG change. While the nutri-GRS explained over 20% of the variability in TG responses to omega-3 fatty acids, the authors concluded that a larger sample size is needed to determine whether these suggestive associations are statistically significant

(Rudkowska et al. 2014). A replication study was then performed in a cohort of Mexican adults, a population from a different ethnic group, and found that the nutri-GRS explained 11% of the variability in TG responsiveness to omega-3 fatty acids ($p < 0.05$). When only the extreme responders were included in the analysis, the nutri-GRS explained up to 29% of the variability ($p < 0.05$).

Fine Mapping of GWAS Signals

Validation of preliminary research findings is important in the field of nutrition in general, including the field of nutritional genomics. Vallée Marcotte et al. built on the work of Rudkowska et al. and aimed to better identify genetic contributors to TG responsiveness by testing associations between genetic variants in the *IQCJ, NXPH1, PHF17*, and *MYB* genes, and TG responses to omega-3 fatty acids in the FAS study (Vallée Marcotte et al. 2016); SNPs in these genes had been suggestively associated with TG responses to omega-3 fatty acids by Rudkowska et al. as indicated above (Rudkowska et al. 2014). In this follow-up study, the researchers aimed to confirm the associated regions using a fine mapping approach. Fine mapping aims to help identify SNPs that are causally associated with a particular effect and also to better refine the prediction potential of identified SNPs. The ultimate goal of this approach is to identify which genetic variations are most likely to be "functional" (Schaid et al. 2018). That is to say, which genetic variations have biological plausibility to be associated with a certain disease/condition, or with a response to nutrition (Schaid et al. 2018). Dense genotyping and imputation are components of the fine mapping approach, alongside cross ethnic mapping, conditional logistical regression for identifying independent associations, and likelihood ratio tests that are used to exclude candidate SNPs that are least likely to be associated with the effect (Edwards et al. 2013).

Significant differences in 13 SNPs of *IQCJ, NXPH1, PHF17*, and *MYB* between responders and nonresponders to omega-3 fatty acids for TG lowering were observed (Vallée Marcotte et al. 2016). Furthermore, significant gene-diet interactions were found for 17 SNPs (Vallée Marcotte et al. 2016). Ultimately, these results provided more evidence that genetic variation in multiple SNPs contribute to the interindividual variability in TG responses to omega-3 fatty acids (EPA + DHA).

Vallée Marcotte et al. then later refined the previously established nutri-GRS (Vallée Marcotte et al. 2019). In this follow-up analysis, again using data from the FAS study, the fine mapping procedure was used in an effort to better identify genetic variations of potential causal influence on the plasma TG response to supplemental omega-3 fatty acids, and also to better refine the prediction potential of identified SNPs. First, dense genotyping and genotype imputation were completed, which led to the identification of 286,149 relevant SNPs. Quality-control tests were then completed, which reduced the number of relevant SNPs to 31,859. From here, tagging SNPs (i.e., SNPs that can be used as surrogate markers of each other – also referred to as SNPs with high linkage disequilibrium) were identified, which led to a total of 505 included SNPs. Next, odds ratios were calculated to identify differences in proportions of nonresponders carrying the minor (less common) allele

of an SNP as compared to responders. A stepwise regression analysis was then run to assess the contribution of SNPs to TG variability from omega-3 fatty acid supplementation and statistically significant SNPs ($p < 0.05$) were included in the calculation of the nutri-GRS.

This approach now explained 50% of the interindividual variability in the plasma TG responses to EPA + DHA. In total, there were 31 SNPs significantly associated with the TG response, and based on these SNPs, a new more refined nutri-GRS was developed (Table 1). Again, similar to the previously discussed work by Rudkowska et al. (Rudkowska et al. 2014), this nutri-GRS can be used to help predict responders, and nonresponders to omega-3 fatty acids (EPA + DHA) for TG change, but with greater accuracy. Individuals with lower scores on the nutri-GRS tool are more likely to be responders, and vice versa for individuals with higher scores (Vallée Marcotte et al. 2019).

While the first analysis was conducted in a Canadian population, this group also replicated these findings in a European population, using data from the FINGEN study, which was a double-blind, placebo-controlled, dose-response cross-over trial (Caslake et al. 2008). They demonstrated that a similar nutri-GRS explained a significant proportion of TG variability to omega-3 fatty acid supplementation (Vallée Marcotte et al. 2019). Given the sum of data, the established 31-SNP nutri-GRS is likely to predict if individuals will be responsive, or not, to omega-3 fatty acid supplementation for TG lowering.

Validation of Nutri-GRS and Contribution of EPA Versus DHA to TG Change

To provide even further validation of the nutri-GRS, the group then tested the GRS prediction potential for TG responsiveness to supplementation with either EPA or DHA in the Comparing EPA to DHA (ComparED) Study. Briefly, participants were randomized to consume either 2.7 g/day of DHA or 2.7 g/day of EPA or 3.0 g/day of control oil (three phases, in random order), and the nutri-GRS was calculated for 122 participants who had abdominal obesity. Ordinal and binary logistic models were used to determine if the GRS predicted plasma TG responsiveness to EPA and/or DHA. Overall, they found that EPA appeared to be driving the identified TG/omega-3 effect on plasma TG (Vallée Marcotte et al. 2020).

Ultimately, when evidence is sufficient to support their use in clinical practice, tools such as nutri-GRSs have the potential to revolutionize the field of precision nutrition, through the provision of more individually targeted nutrition advice, based on multiple SNPs.

Importance of Understanding Biological Plausibility

While it is fascinating to observe that genetic variations are associated with diseases/conditions and/or health-related responses to nutrition, there is the

Table 1 31-SNP nutri-GRS

Gene, rs number	Alleles[a]	Associated points
IQCJ-SCHIP1, rs7639707	A/G	+1
IQCJ-SCHIP1, rs62270407	C/T	−1
NXPH1, rs61569932	G/T	+1
NXPH1, rs1990554	A/C	+1
NXPH1, rs6463808	A/G	+1
NXPH1, rs6966968	A/G	+1
NXPH1, rs28473103	A/G	−1
NXPH1, rs28673635	A/G	+1
NXPH1, rs12702829	C/T	+1
NXPH1, rs78943417	A/T	−1
NXPH1, rs293180	G/T	+1
NXPH1, rs1837523	C/T	−1
PHF17, rs1216346	C/T	+1
PHF17, rs114348423	A/G	+1
PHF17, rs75007521	G/T	−1
MYB, rs72560788	C/T	−1
MYB, rs72974149	A/G	−1
MYB, rs210962	C/T	−1
MYB, rs6933462	C/G	+1
NELL1, rs79624996	A/G	+1
NELL1, rs1850875	C/T	+1
NELL1, rs78786240	C/T	−1
NELL1, rs117114492	G/T	+1
SLIT2, rs184945470	C/T	+1
SLIT2, rs143662727	A/G	−1
SLIT2, rs10009109	C/T	+1
SLIT2, rs10009535	A/G	+1
SLIT2, rs61790364	A/G	+1
SLIT2, rs73241936	C/T	+1
SLIT2, rs16869663	A/G	+1
SLIT2, rs76015249	A/G	+1

Adapted with permission from: Vallée Marcotte et al. 2019
For individuals carrying one or two minor alleles, provide the associated number of points (either +1 or −1). For individuals homozygous for the major allele, provide 0 points. Count the overall number of points. Individuals with lower nutri-GRS are more likely to respond to approximately 3.0 g/day EPA + DHA for TG lowering
[a]Minor alleles are underlined

possibility that these findings may have occurred due to chance alone (although robust statistical analyses help mitigate this concern). If we can determine that a particular genetic finding is biologically plausible, then we increase our confidence that a true effect exists (Van der Velden et al. 2011). Various methods exist to give us insights into the biological plausibility (or lack thereof) in genetics and nutritional genomics such as transcriptomics (gene expression), proteomics,

metabolomics (Romero et al. 2006), or the fine mapping approach mentioned previously (Schaid et al. 2018). An in-depth discussion of these various omics approaches goes beyond the scope of this chapter, but it is important to understand that when a nutrigenetic finding is biologically plausible, the scientific validity of this finding is enhanced (Strande 2017). For example, Vallée Marcotte et al. explored the biological plausibility of SNPs within the *IQCJ, NXPH1, PHF17*, and *MYB* genes that have been associated with plasma TG responses to omega-3 fatty acid supplementation. There was evidence of biological plausibility through DNA methylation and gene expression, providing further validity for the nutri-GRS. However, they still concluded that more research is needed to better understand the relationship between *IQCJ, NXPH1, PHF17*, and *MYB* genes and TG metabolism (Vallée Marcotte et al. 2017).

Conclusion

Overall, it is well recognized that individuals have different health responses to the same nutritional interventions or exposures. Genetic variation can help explain why this phenomenon occurs; this science can be referred to using the umbrella term *nutritional genomics*. GWAS and GWAIS approaches can be used to help better understand how multiple SNPs may collectively influence health responses to nutrition.

Other Applications of GWAS and GWAIS for Precision Nutrition

In this chapter, the GWAS approach for plasma TG responsiveness to omega-3 fatty acids was thoroughly discussed, but it is important to note that GWAS and GWAIS approaches can be (and have been) applied to a number of other applications in precision nutrition. Many of the findings from other GWAS and GWAIS require further research, and replication before the findings may be applicable to clinical practice. For example, using data from the UK Biobank, researchers have used the GWAIS approach in an observational study to identify other genetic variations that may contribute to the impact of fish oil supplementation on plasma lipid levels, including TG. They found a significant interaction between *GJB6-GJB2-GJA3* rs112803755, use of fish oil supplementation, and plasma TG levels (Francis et al. 2021). Moreover, Coltell and colleagues conducted a GWAIS to determine if adherence to a Mediterranean diet altered the impact of genetic variation on serum bilirubin levels. They found 15 SNPs with significant gene-diet interactions, including SNPs in the *IL17B, LAMA2, LOC107985179, EDNRA, LINC01541*, and *LINC01331* genes. Notably, this group completed a sex-stratified analysis to determine if genetic variation contributing to serum bilirubin concentrations may be modulated by adherence to a Mediterranean diet; no sex-specific gene-diet interactions were found (Coltell et al. 2019). In this case, the sample size may have been too small to detect any significant gene-diet interactions (Coltell et al. 2019), but

nonetheless, sex-stratified analyses are important for genetic and nutrigenetic research given that the impact of genetic variation and nutrition on health may vary depending on sex (Corella et al. 2018).

The potential applications of GWAS and GWAIS for nutritional genomics and precision nutrition are broad. Other groups have explored topics such as the influence of genetic variation on: liver cirrhosis dependent on alcohol intake (Emdin et al. 2021), serum alpha-tocopherol levels after vitamin E supplementation, Parkinson's disease risk dependent on caffeine intake (Hamza et al. 2011), and TG levels dependent on alcohol intake (Tan et al. 2012) using these approaches. The possibilities for the GWAS and GWAIS approaches to be applied to precision nutrition research are endless.

Mini-Dictionary of Terms

- **Biologically Plausible.** Evidence of a mechanism of action; biologically "possible" or "believable."
- **Functional SNPs.** SNPs that have demonstrated an impact on protein function, and thus physiological processes.
- **Gene.** The basic functional unit of heredity, which contains a sequence of nucleotides.
- **Genome-Wide Association and Interaction Study (GWAIS).** A GWAIS is similar to a GWAS but adds an additional layer – an interaction with an environmental component, such as nutritional intake. A GWAIS studies the interactions between genetic variation and health outcomes, based on an environmental/lifestyle factor – in this case, dietary intake.
- **Genome-Wide Association study (GWAS).** A GWAS is a type of study in which genetic markers are scanned in a complete set of DNA in an effort to identify which genetic markers contribute to a specific disease, condition, phenotype, or response to a nutrition intervention.
- **Genotype.** An organism's genetic characteristics.
- **Nutri-Genetic Risk Score (Nutri-GRS).** A nutri-GRS is a tool that can be used to "score" the risk of a health outcome, dependent on nutritional intake. For example, a nutri-GRS has been developed to help predict an individual's TG response to EPA + DHA supplementation; individuals with lower risk scores tend to have greater TG-lowering responses to EPA + DHA.
- **Nutrigenetics.** A science that explores connections between genotype, and the response to nutritional intake, on health/disease outcomes.
- **Nutrigenomics.** A science that explores the impact of nutrition on gene expression and thus protein and metabolite levels.
- **Nutritional Genomics.** An umbrella term encompassing both nutrigenetics and nutrigenomics.
- **Single Nucleotide Polymorphism (SNP).** The most common type of human genetic variation, whereby one single nucleotide is substituted with another.

Key Facts of GWAS and GWAIS for Nutritional Genomics and Precision Nutrition

- Precision nutrition recognizes that the one-size fits all approach to nutrition is not optimal, and multiple factors such as genetics, socioeconomic status, physical activity levels, the gut microbiome, and others, contribute to nutritional needs.
- Interindividual variability in health outcomes to nutrition are widespread and can be exemplified in the TG responses to EPA + DHA; about 30% of people exhibit no change in TG or increases in TG in response to EPA + DHA supplementation, while about 70% exhibit a TG reduction.
- Genetic variation can help to explain this interindividual variability observed in responses to diet.
- GWAIS stands for genome-wide association and interaction study, and this type of study is used to identify multiple SNPs that contribute to a particular health outcome, dependent on an environmental factor such as nutritional intake/exposure; it includes a gene-diet interaction term in the statistical analysis and is typically conducted within an observational study.
- GWAS stands for genome-wide association study, and this type of study can be used to identify how responses to a nutrition intervention are linked to genetic variation; there is typically a nutrition intervention component in GWAS relevant to nutrigenetics.
- One of the most robustly studied topics using the GWAS approach is the TG response to omega-3 fatty acids (EPA + DHA).
- 31 SNPs in the *IQCJ, NXPH1, PHF17, NELL1, SLIT2*, and *MYB* genes can explain a large portion of the interindividual variability in plasma TG responses to supplemental EPA + DHA.
- When a gene-diet interaction has been demonstrated to be biologically plausible, we increase our confidence in the scientific validity of this interaction. Several of the abovementioned SNPs have demonstrated biological plausibility.

Summary Points

- Individuals respond differently (in terms of health outcomes) to the same nutrition interventions/exposures.
- Genetic variation plays a key role in the interindividual variability in health responses to various nutrition interventions/exposures.
- Multiple SNPs may influence these variable responses; the GWAS and GWAIS approaches can integrate genetic risk and nutritional intake data to help identify which SNPs may play a role in this interindividual variability.
- While many GWAS and GWAIS still require replication and further research, multiple robust studies have been conducted exploring genetic variability, omega-3 fatty acids supplementation, and TG responses using these approaches.
- Based on this research, a 31-SNP nutri-GRS has been developed, which can be used to predict whether or not individuals may be responsive to EPA + DHA for TG lowering; this research is generalizable to adults with overweight/obesity.

- Overall, GWAS and GWAIS can help us to better understand why individuals respond differently to the same nutrition intervention and can be used to develop nutri-GRSs (particularly the GWAS approach). These nutri-GRSs can then be used to help target dietary advice for the individual.

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The Alpha-Klotho Gene as an Anti-ageing Biomarker: Measures and Applications to the Effects of Nutrition

28

Lucas Jurado-Fasoli

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Abstract

Ageing is a complex process characterized by a progressive decline of physiological functions, which leads to an impaired physical integrity and an increase of mortality risk. Specifically ageing is influenced by both genetic and

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environmental factors such as nutrition or physical activity. Recently, different specific ageing biomarkers have been proposed to provide a better insight of the ageing process. One of these new ageing biomarkers is the Klotho gene which encodes different Klotho proteins with anti-ageing physiological and metabolic functions. Therefore, we discuss here the potential mechanisms of the influence of nutrition on S-Klotho levels. Later, we review the current evidence about nutrition and S-Klotho in both preclinical models and humans, as well as future investigation to yield evidence into the influence of nutrition on ageing, specifically on S-Klotho.

Keywords

Ageing · Longevity · Nutrition · Diet · Dietary compounds · Lifespan · Alcohol · Dietary inflammatory index · Bioactive compounds · Vitamin D · Caloric restriction · Oxidative stress

Abbreviations

ADF	Alternate day fasting
AMPk	Adenine monophosphate protein kinase
CRM	Caloric restriction mimetics
DASH	Dietary Approaches to Stopping Hypertension
DII	Dietary inflammatory index
EPA	Eicosapentaenoic acid
FGF	Fibroblast growth factors (FGF)
FMD	Fasting-mimicking diet
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor I
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NaK-ATPase	Sodium–potassium adenosine triphosphatase
NPT2A	Type II sodium-dependent phosphate cotransporter
NPT3	Na (+)-phosphate cotransporter 3
ROMK1	Renal outer medullary potassium 1
TGF-β	Transforming growth factor-β
TRE	Time-restricted eating
TRPC6	Transient receptor potential canonical type 6
TRPV5	Transient receptor potential channel vanilloid 5
VDR	Vitamin D receptor

Introduction

The Klotho gene, named after one of the three Fates in Greek mythology, the goddess who spins the thread of life, is involved in the ageing phenotype. It was identified in 1997 by Kuro-O et al. as a gene lacking in mice with multiple disorders

resembling human ageing and with a shortened lifespan (Kuro-o et al. 1997). Conversely, the Klotho overexpression acts as an anti-ageing gene that lengthen lifespan (Kurosu et al. 2005). This Klotho gene encodes a single-pass transmembrane protein which is mainly expressed in the distal convoluted tubules of kidneys (Kuro-o et al. 1997).

Of note, three different Klotho-related genes have been recognized. Firstly, the α-Klotho which is expressed in distal convoluted tubules in the renal cortex, the parathyroid glands, and the choroid plexus in the brain (Kuro-o et al. 1997). The α-Klotho is responsible for mainly controlling the mineral homeostasis (Kuro-o et al. 1997). Secondly, the β-Klotho which is expressed in the liver, the endocrine pancreas, the adipose tissue, and the brain (Urakawa et al. 2006; Hu et al. 2010). Its main function is to influence bile acids, lipid, and energy metabolism together with the fibroblast growth factors (FGF) 15/19 and 21 (Urakawa et al. 2006; Hu et al. 2010). Lastly, the γ-Klotho which is a half-size Klotho-related gene expressed in brown adipose tissue (Fon Tacer et al. 2010; Kim et al. 2015).

Concretely, the α-Klotho gene expresses three different proteins: the intracellular protein which binds NaK-ATPase; the cell-membrane protein which forms a complex with FGF receptors and FGF23; and the secreted form from the extracellular domain into blood, plasma, urine, and cerebrospinal fluid (Imura et al. 2004). The release of S-Klotho in the peripheral circulation reaches different tissues and exerts distinct actions (Kuro-o et al. 1997; Cheikhi et al. 2019; Kuro-o 2019). Although the secreted form shows an accurate and strong relationship with the α-Klotho gene expression (Saghiv et al. 2017), the membrane and the secreted form of Klotho (S-Klotho), exert different physiological functions.

These actions mainly include the regulation of the endothelial integrity (Dalton et al. 2017), the modulation of the endothelial nitric oxide synthesis (Saito et al. 1998; Nagai et al. 2000), and the modulation of the action of different hormones and molecules such as insulin, insulin-like growth factor I, transforming growth factor-β (TGF-β), Wnt signaling, and gamma interferon operating as a paracrine and/or endocrine mediator (Dalton et al. 2017; Kuro-o 2019). In sooth, previous evidence has proved that α-Klotho can suppress the insulin and Wnt signaling pathways, inhibit oxidative stress, and regulate phosphatase and calcium absorption (Xu and Sun 2015). Mechanistically, S-Klotho can regulate the activity of diverse ion channels and transporters such as the TRPV5 and ROMK1 (Kuro-o 2019). Additional information about the specific functions of S-Klotho could be found in Fig. 1.

Interestingly, high levels of S-Klotho have been related to a better health status in humans and with a less prevalence of age-related diseases. Indeed, high levels of S-Klotho have been associated with a healthier body composition (i.e., lower adiposity, higher lean mass and bone mineral density) (Amaro-Gahete et al. 2019b), and metabolic flexibility during basal and exercise conditions (i.e., higher fat oxidation during both conditions) (Amaro-Gahete et al. 2019c). Importantly, high levels of S-Klotho in humans have been demonstrated to be related with a lower risk of cardiovascular disease (Semba et al. 2011a) and all-cause mortality (Semba et al. 2011b). Together, S-Klotho may be considered

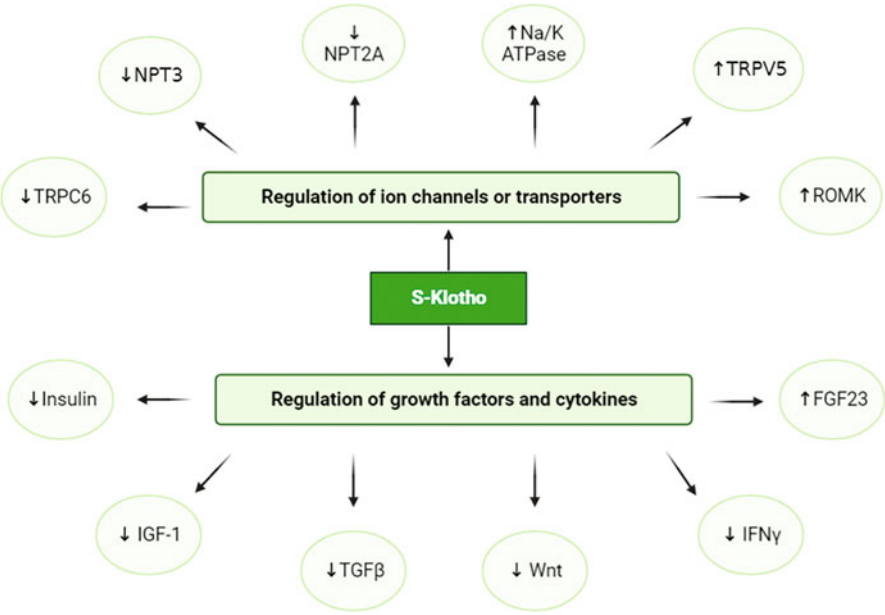

Fig. 1 Main S-Klotho physiological functions
This schematic summarizes the main physiological and molecular functions of S-Klotho. Abbreviations: *TRPC6* Transient receptor potential canonical type 6, *NPT3* Na (+)-phosphate cotransporter 3, *NPT2A* type II sodium-dependent phosphate cotransporter, *TRPV5* Transient receptor potential channel vanilloid 5, *ROMK* renal outer medullary potassium channel, *IGF-1* insulin-like growth factor I, *TGF-β* transforming growth factor-β, *IFN-γ* Interferon-γ, *FGF-23* fibroblast growth factor 23

a potential marker of different chronic diseases such as cancer (Zhou and Wang 2015), chronic kidney disease (Castaño and Maurer 2017), the severity of coronary disease (Navarro-González et al. 2014), and type II diabetes (Nie et al. 2017) among others (Figs. 2, 3, and 4).

Ageing and age-related chronic diseases may be influenced by different lifestyle interventions, mainly physical activity or exercise interventions, dietary interventions, psychological interventions, and sleep management interventions. Notably, dietary interventions can influence ageing and chronic diseases during the life course and adulthood (Shlisky et al. 2017). In this sense, the Mediterranean diet, caloric restriction, caloric restriction mimetics, and some anti-ageing dietary patterns have been proposed as potential interventions to increase human longevity (Chrysohoou and Stefanadis 2013).

On the other hand, exercise training has demonstrated to increase S-Klotho levels (Amaro-Gahete et al. 2019a), and a good sleep quality has been related to higher levels of S-Klotho in humans (Mochón-Benguigui et al. 2020). However, little is known about the influence of nutrition on S-Klotho levels. Therefore, the purpose of this chapter is to examine the current evidence of the relationship between nutrition and S-Klotho plasma levels.

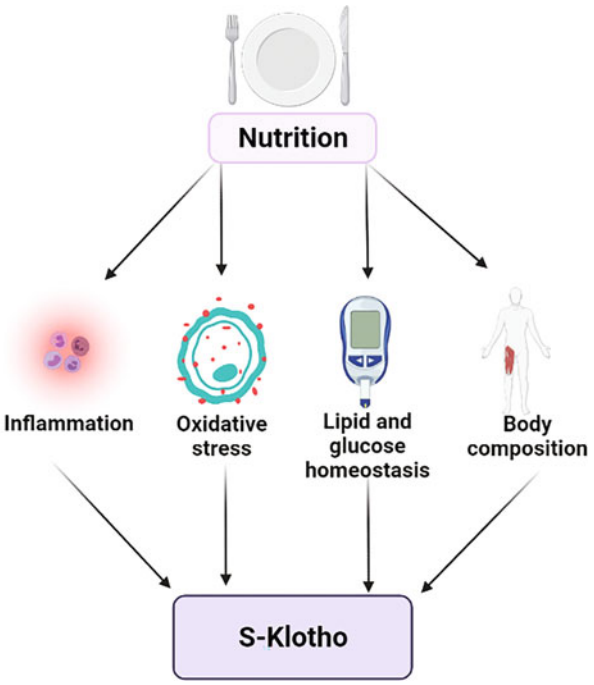

Fig. 2 Plausible mechanisms of the effect of nutrition on S-Klotho
This schematic summarizes the main possible mechanisms of the effect of nutrition on S-Klotho plasma levels.

Plausible Mechanisms of the Effect of Nutrition on S-Klotho Levels

The S-Klotho plasma levels are considered a powerful longevity biomarker (Kuro-o et al. 1997), partially because of their different anti-ageing functions, such as the reduction of inflammatory processes (Kim et al. 2015) or a decrease in cellular oxidative stress (Yamamoto et al. 2005). It has been previously proposed that nutrition or dietary compounds could modulate S-Klotho through different physiological mechanisms.

Firstly, both the acute and the chronic inflammatory states can regulate S-Klotho levels (Izquierdo et al. 2012). Secondly, a high production of reactive oxygen species which turns into oxidative stress can decrease α-Klotho expression (Mitobe et al. 2005). Thirdly, both lipid and glucose impairments can downregulate α-Klotho expression (Hu et al. 2013). Lastly, body composition, specifically skeletal muscle, is a key regulator of the anti-ageing protein S-Klotho (Avin et al. 2014). Together, S-Klotho plasma levels could be regulated through a wide range of physiological functions that also encompass pathological processes involved in ageing and chronic diseases. Importantly, it is widely known that nutrition and dietary compounds can influence all the abovementioned physiological processes, such as inflammation, cardiometabolic parameters, or body composition. Therefore, it is plausible that nutrition and dietary compounds could be an anti-ageing strategy through the modulation of S-Klotho levels.

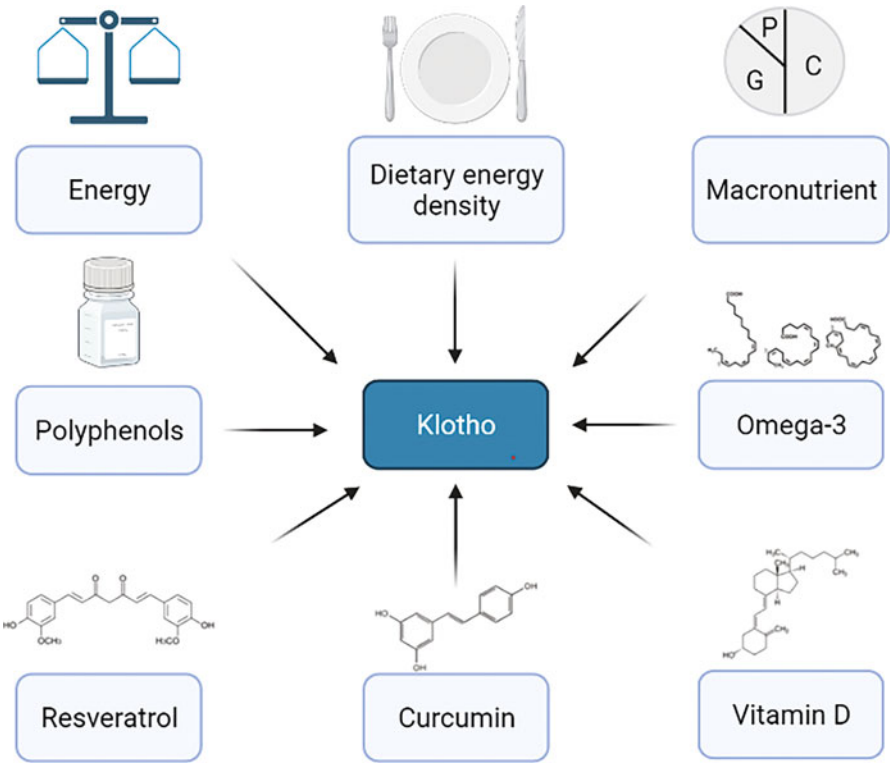

Fig. 3 Summary of the main nutritional determinants of Klotho in preclinical models
This schematic summarizes the potential nutritional factors that have been demonstrated to influence Klotho in preclinical models

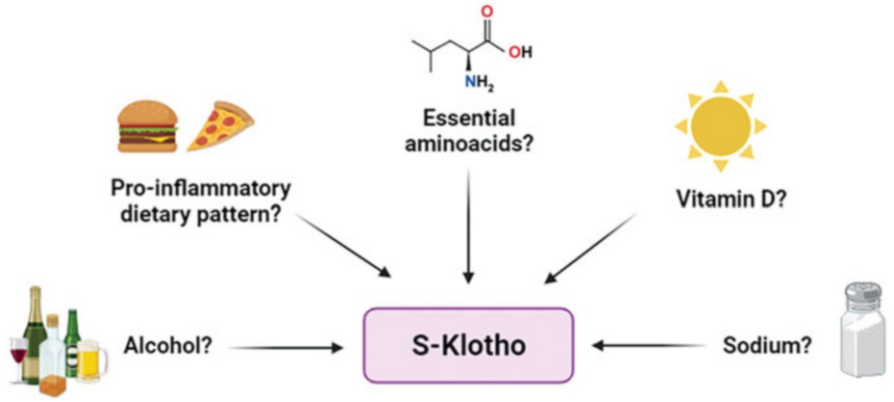

Fig. 4 Summary of the plausible nutritional determinants of Klotho in humans
This schematic summarizes the potential nutritional factors that may modulate S-Klotho in humans

Effect of Nutrition in Klotho: Evidence from Preclinical Models

Several studies have shown that dietary intake, specific nutrients, or foods can modulate the α-Klotho gene expression in cellular or animal models and the S-Klotho levels.

Dietary Energy

Caloric restriction is the main dietary strategy that has been demonstrated to promote longevity in monkeys, rats, and mice (Acosta-Rodríguez et al. 2021). In this sense, 4 weeks of a 60% of caloric restriction increased α-Klotho expression in male rats (Miyazaki et al. 2010). The increment in Klotho expression was inversely related to changes in serum zinc levels, suggesting that this micronutrient might influence the regulation of Klotho during a hypocaloric period. In addition, a lower caloric restriction based on the reduction of 30% of the energy intake (mainly from carbohydrates) has also been effective to upregulate Klotho expression in mice (Schafer et al. 2015). Hence, it seems plausible that caloric restriction can increase lifespan partially through the restoration of Klotho levels in preclinical models.

Recently, dietary strategies that restrict the timing of food intake have emerged as potential alternatives to caloric restriction. Specifically, intermittent fasting has been demonstrated to increase lifespan in mice and rats and time restricted-eating has appeared as a new strategy that could have the benefits of food restriction and circadian timing synchronization (Acosta-Rodríguez et al. 2021). A recent study in rats has shown that intermittent fasting in the form of every-other-day fasting can increase S-Klotho levels in old mice, but not in middle or young mice (Badreh et al. 2020). This every-other-day fasting also upregulated Klotho expression in comparison to ad libitum and caloric restriction diets in mice (Pereira et al. 2021). Of note that this upregulation also improved cognition in mice, suggesting the Klotho upregulation as a potential mechanism for these cognitive improvements driven by intermittent fasting. Altogether, these studies put forward intermittent fasting as a potential strategy to increase lifespan, partially through the increment in S-Klotho levels.

Together with food timing, dietary energy density plays an important role in the regulation of energy intake. Energy-dense diets decreased renal Klotho with the consequent increment in circulating levels of FGF23 (Rodriguez and Aguilera-Tejero 2018). Mechanistically, an energy-dense diet (especially high-fat diets) can decrease renal Klotho, and renal Klotho/actin ratio mainly through the P retention in rats (Raya et al. 2016). However, energy restriction strategies are difficult to sustain long term, and compliance is low. Thus, the manipulation of the intake of different macronutrients could be an effective strategy to increase lifespan in different animal models rather than reducing overall energy intake (Acosta-Rodríguez et al. 2021).

Dietary Macronutrients

Specifically, a high-fat diet could have a life-shortening effect in preclinical models, as it has been demonstrated in several studies where animal models reduced the expression of α-Klotho after following a high-fat diet (Yoshikawa et al. 2018; Shafie et al. 2020). Consequently, this decrement in α-Klotho after a high-fat diet may have different physiological harms in terms of cardiovascular alterations or cognitive alterations (Yi et al. 2016; Shafie et al. 2020). Although ketogenic diets have been postulated as a potential strategy for ageing and longevity (Acosta-Rodríguez et al. 2021), it seems that a high-fat, low-carb diet may not have a positive role in the regulation of Klotho, at least in preclinical models.

Another modification of dietary composition which has been studied is protein restriction. However, a high protein diet increased α-Klotho expression in mice, suggesting a potential anti-ageing role of a high-protein and low-calorie diet in mice (Shafie et al. 2020).

Beyond energy and macronutrient manipulation in the context of ageing, several specific nutrients have been postulated to influence hallmarks of ageing and to increase lifespan.

Evidence about the potential role of omega-3 fatty acids on the regulation of Klotho in animal models is controversial. Particularly, in Klotho mutant mice, EPA reduced arterial calcification, decreased oxidative stress, and downregulated NADPH oxidase-4 expression and activity (Nakamura et al. 2017). Conversely, fish oil supplementation did not modify α-Klotho expression in a model of chronic kidney disease (Agudelo et al. 2018). Due to the marked differences in the experimental design of these studies, more research is needed to elucidate the potential role of omega-3 in the modulation of Klotho.

Micronutrients

Mechanistically, the active form of vitamin D induces the expression of α-Klotho, due to the vitamin D-responsive elements present in the promoter of α-Klotho (Kuro-o 2019). Indeed, 1,25-vitamin D treatment upregulated the expression of membrane and secreted forms of Klotho in both human and mouse cell lines suggesting a potential role of vitamin D in the secretion of circulating Klotho (Haussler et al. 2012).

Interestingly, S-Klotho functions as a phosphaturic factor independently of FGF23 which directly influences the development of ageing-like symptoms (Kuro-o 2019). In line with this, a higher dietary phosphate intake during 7 days decreased α-Klotho expression in mice (Fukuda-Tatano et al. 2019). These findings are supported by another study that also demonstrated that α-Klotho expression is inversely related to the level of dietary phosphate (Morishita et al. 2001). These studies suggest that ageing progression may be partially modulated by dietary phosphate through α-Klotho expression, at least in mice.

Numerous bioactive compounds have been widely studied as potential regulators of oxidative stress and inflammatory status in the ageing context. Interestingly, curcumin can upregulate membrane Klotho in mouse cells due to its non-vitamin-D VDR ligand activity (Haussler et al. 2012). Other bioactive compounds such as resveratrol (Hsu et al. 2014), magnesium, or polyphenols (Buchanan et al. 2020) upregulated Klotho expression in cell and animal models. However, there is a lack of high-quality mechanistic studies focusing on dietary bioactive compounds and Klotho levels, and more evidence will help to understand the role of these compounds on ageing.

Effect of Nutrition in Klotho: Human Studies

Despite the relative abundance of pre-clinical evidence suggesting the potential role of nutrition in the modulation of S-Klotho plasma levels, there is a lack of solid evidence investigating the influence of nutrition on S-Klotho in humans, and most of the studies have an observational design.

Observational Evidence

Notably, different studies have reported the relationship between alcohol consumption and S-Klotho in different populations. Higher consumption of alcoholic drinks was associated with lower levels of S-Klotho plasma levels in middle-aged adults (Jurado-Fasoli et al. 2019a), whereas ethanol intake was positively associated with S-Klotho plasma levels in young healthy women, but not in men (Jurado-Fasoli et al. 2021). The discrepancies between these studies could be due to differences in the age of the populations and differences in the dose of alcohol ingested. Conversely, González-Reimers et al. observed a direct correlation between ethanol intake and S-Klotho plasma levels in heavy alcoholic men, especially in non-cirrhotic individuals (González-Reimers et al. 2019). Additionally, S-Klotho levels have been demonstrated to be increased in cirrhotic patients, especially in alcoholics, suggesting a potential role of this biomarker in liver impairment and cardiovascular disease (Quintero-Platt et al. 2017; González-Reimers et al. 2018). Mainly, the inflammatory effects of S-Klotho have been hypothesized to be the plausible mechanisms that might explain these associations, since it could be increased to compensate the pro-inflammatory status derived from an abusive alcohol intake.

In turn, the dietary inflammatory index (DII) has been recently developed as a dietary tool to identify the inflammatory potential of the diet. In this sense, a pro-inflammatory dietary pattern determined with the DII was positively associated with S-Klotho plasma levels in middle-aged adults (Jurado-Fasoli et al. 2020) but was inversely associated with S-Klotho plasma levels in young adults (Jurado-Fasoli et al. 2021). Of note, these associations were modulated by body composition, mainly lean mass. These differences could be due to the age-related differences between studies, suggesting that in young adults a pro-inflammatory dietary pattern could decrease S-Klotho levels, whereas in an older population such as middle-aged

adults a pro-inflammatory dietary pattern could increase S-Klotho plasma levels as a compensatory mechanism to reassemble the inflammatory status.

As we have commented, the dietary pattern could have an important influence on ageing, specifically on S-Klotho levels. However, adherence to the Mediterranean diet, a well-known healthy dietary pattern, was inversely related to S-Klotho plasma levels (Jurado-Fasoli et al. 2019b). However, this finding could be explained by the higher consumption of nuts in those participants with low adherence to the Mediterranean diet. Nuts are rich in α-linolenic acid, an omega-3 fatty acid that can be transformed into other omega-3 fatty acids. Therefore, since mechanistic evidence has shown a potential positive relationship between omega-3 and S-Klotho levels, it seems plausible that nuts consumption could have an influence on S-Klotho levels in humans. Conversely, all these results were dependent on the body composition of the participants, specifically on the lean mass (Jurado-Fasoli et al. 2019b).

Intervention Studies

Beyond the observational studies performed in humans, only a few studies have investigated the role of nutrition con Klotho levels in humans. Protein manipulation has been one dietary modification studied for increasing lifespan. In this sense, 14-weeks of a low-protein diet (0.6 g/kg/day) decreased S-Klotho levels in 42 patients non-diabetic with chronic-kidney disease (Milovanova et al. 2018). Interestingly, patients who followed the low-protein diet and were supplemented with ketoanalogues of essential amino acids (0.1 g/kg of body weight/day did not modify their S-Klotho plasma levels after 14 weeks and their levels were higher at the end of the study than those who only followed the low-protein diet. This study may suggest the importance of providing adequate amounts of essential amino acids to prevent an inadequate nutritional status which can influence ageing biomarkers.

As we have previously commented, there is a tight mechanistic relationship between S-Klotho and vitamin D. Indeed, 12 weeks of 25-OH vitamin D supplementation (50,000 IU/week) in 90 elderly participants was demonstrated to prevent the age-related reduction in S-Klotho levels in comparison to the placebo group (Jebreal Azimzadeh et al. 2020). This study may suggest that vitamin D supplementation could be a plausible effective strategy to prevent the physiological decline during ageing.

In contrast, a previous study investigated the effects of oat consumption on renal biomarkers in patients with chronic kidney disease. In this study, S-Klotho was measured as a marker of kidney function; however, after 8 weeks of intervention based on the consumption of 50 g/day + dietary recommendations, circulating levels of S-Klotho did not change (Rouhani et al. 2018).

Besides the main anti-ageing functions of S-Klotho, this biomarker is involved in sodium reabsorption and in the regulation of blood pressure. A previous cross-over clinical trial compared the effects of a 7-day low-Na^+ diet (3 g/day NaCl) vs. 7-day high-Na^+ diet (29 g/day NaCl). In this sense, S-Klotho levels increased after the low-salt diet whereas decreased after the high-salt intake (Hu et al. 2020). Interestingly, the S-Klotho response to salt intake was dependent on the salt sensitivity of the

participants, being more pronounced in those salt-resistance participants (Hu et al. 2020). This study suggests that salt consumption could contribute to the development of hypertension partially due to its influence on S-Klotho levels. Indeed, it puts forward the idea that not only energy or macronutrient intake is important, also dietary components added to the food while cooking such as salt.

Taking all together, human studies have shown that the relationship between nutrition and S-Klotho levels exists. However, it is worth mentioning that there is a lack of high-quality evidence to establish the specific relationship between diet and this novel biomarker. Therefore, high-quality clinical trials investigating the role of different types of diets, foods, dietary patterns, or dietary bioactive compounds are needed to provide dietary guidance for achieving healthy ageing.

Future Investigations into S-Klotho

Since 1997, different studies have investigated the role of Klotho in ageing. One field of research has been the influence of nutrition on both Klotho expression and S-Klotho levels. However, the evidence is still scarce. Moving forward, it will be important to elucidate the effects of different nutritional components on S-Klotho levels, specifically in human studies.

Fasting Strategies

Caloric restriction can increase lifespan in different animal models, due to the reduction in the energy consumed, the influence in the circadian system, and the extended periods of fasting. Indeed, there is a tight relationship between circadian clocks and ageing molecular targets such as sirtuins, AMPk, or mTOR (Acosta-Rodríguez et al. 2021). In this sense, different dietary interventions have been proposed to influence anti-ageing and pro-ageing pathways, partially due to the circadian influence in the nutrient-sensing pathways (Acosta-Rodríguez et al. 2021). These dietary interventions involve different fasting strategies such as time-restricted eating (TRE), alternate day fasting (ADF), periodic fasting such as the 5:2 fasting, or fasting-mimicking diets (FMD). Although these strategies could protect against chronic diseases (i.e., obesity or cardiometabolic diseases), their role in promoting lifespan has not been investigated. Therefore, to understand the potential role of these dietary strategies in the ageing process it is essential to investigate their influence on S-Klotho levels in humans to elucidate if their positive effects may partially due to the restoration of the levels of this biomarker.

Dietary Compounds

Different dietary bioactive compounds have been defined as caloric restriction mimetics (CRM), due to their influence in similar molecular pathways to caloric

restriction with the benefit of no limiting food intake. Some of these dietary compounds have been investigated to increase α-Klotho expression in preclinical models such as curcumin or resveratrol (Haussler et al. 2012; Hsu et al. 2014). However, there is a lack of evidence in humans that supports their use to increase lifespan. Therefore, well-designed double-blind clinical trials are needed to investigate the effects of dietary compound supplementation on S-Klotho levels and others ageing markers. Some of the potential CRM dietary compounds to be investigated could be astaxanthin, catechin, epigallocatechin gallate, fisetin, tyrosol, rosmaniric acid, quercetin, naringenin, and kaempferol, among others (Vaiserman et al. 2021).

Dietary Patterns

Several studies have shown that concrete dietary patterns are potentially related to longevity. In this sense, the five populations with a high prevalence of centenarians have been described as the "Blue Zones" in Okinawa (Japan), Sardinia (Italy), Loma Linda (California), Nicoya (Costa Rica), and Ikaria (Greece). To note, these populations have a plant-based dietary pattern, as well as the Mediterranean dietary pattern and the DASH dietary pattern. However, despite the wide-known positive effects in health-related outcomes such as the prevention of chronic diseases and the lifespan extension of the above-mentioned diets, there is no evidence investigating the effects of long-term dietary patterns in S-Klotho levels. Therefore, it is essential to design long-term interventions based on different dietary patterns to elucidate the potential anti-ageing effects specifically in this biomarker.

Conclusion

In conclusion, different dietary components have been demonstrated to influence α-Klotho expression in preclinical models whereas other dietary components have been associated with S-Klotho levels in observational studies in humans. However, only a few intervention studies in humans have elucidated the effects of different diet interventions in S-Klotho levels. Taken all together, this chapter shows that S-Klotho levels could be modulated through diet both in preclinical models and humans. Further understanding of the effect of dietary components on S-Klotho levels is important to establish nutritional recommendations for the ageing process.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter we review the influence of nutrition on S-Klotho levels in both preclinical models and in humans. In particular, the studies included a wide variety of nutritional outcomes in the assessment of their relationship with S-Klotho levels. Preliminary studies suggest that S-Klotho could be modulated through nutrition in preclinical and human studies. However, findings from these studies are suitable for

other chronic diseases where there is an exacerbated pro-inflammatory status such as obesity, cardiometabolic diseases, or cancer. It is important to highlight that the vast majority of these studies were observational or in preclinical models. Therefore, the application of these interventions in a clinical field is needed to evaluate the effects of nutrition on ageing, chronic diseases, and specifically on S-Klotho levels.

Mini-Dictionary of Terms

- ***α-Klotho***. *The protein encoded by the α-Klotho gene which forms a complex with FGF23 and sheds S-Klotho into blood, urine, and cerebrospinal fluid.*
- ***S-Klotho***. *The extracellular domain of the α-Klotho protein which elicits an autocrine, paracrine, or endocrine anti-ageing activity.*
- ***FGF23***. *Protein which is required to form the FGF23–αKlotho–FGFR1c complex which is responsible for the shedding of S-Klotho.*
- ***Dietary inflammatory index***. *Dietary tool that determines the inflammatory potential of the diet (pro-inflammatory or anti-inflammatory).*
- ***Caloric restriction mimetics***. *Bioactive compounds that activate the same metabolic pathways than caloric restriction without decreasing food intake.*
- ***Dietary pattern***. *Quantity, variety, and combination of different foods and beverages in the context of a diet, and the frequency of their habitual consumption. It considers synergistic and antagonistic interactions among nutrients, and the different sources of a same nutrient.*

Key Facts of Klotho

The Klotho gene was identified in 1997 by Kuro-O et al. as a gene lacking in mice with multiple disorders resembling human ageing and with a shortened lifespan.

Three different Klotho genes have been recognized: α-Klotho, β-Klotho, and γ-Klotho.

The α-Klotho gene expresses the intracellular, the cell membrane, and the secreted form of the extracellular proteins.

The secreted form of α-Klotho protein exerts different physiological actions through its endocrine, paracrine, and autocrine activities.

The main functions of S-Klotho are the regulation of ion channels or transporters and the regulation of growth factors and cytokines.

Summary Points

- *Nutrition may influence S-Klotho levels through the modulation of the inflammation, the oxidative stress, the lipid and glucose homeostasis, and the body composition.*

- *Energy, dietary energy density, macronutrients, omega-3, and micronutrients (vitamin D, curcumin, resveratrol, and polyphenols) have demonstrated to modulate Klotho expression in preclinical models.*
- *There is a lack of evidence about the relationship between nutrition and S-Klotho levels in humans.*
- *Observational studies in humans suggest that alcohol consumption and a pro-inflammatory dietary pattern may influence S-Klotho levels.*
- *Intervention studies in humans show that essential amino acids, vitamin D, and sodium are some dietary components that may modulate S-Klotho levels.*
- *Future studies are needed to establish scientific recommendations regarding the nutritional influence on ageing, specifically on S-Klotho levels.*

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Part V

Functional and Physiological Variables and Platforms

BMI as a Biomarker in Patients' Nutritional Assessment

29

Jacek Budzyński and Beata Szukay

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Abstract

Body mass index (BMI) is widely used as a first-line screening biomarker for nutritional status assessment. The advantages of BMI are its simplicity, low cost, and non-invasiveness. However, this biomarker has a number of limitations, which lead to low sensitivity in the diagnosis of both malnutrition and obesity; for example, more than half of the people with a high percentage of body fat (e.g., >30%) are diagnosed as being in the BMI range for a normal weight. The shortcomings of BMI as a biomarker of malnutrition depend on: (a) the slow effect of decreased food intake on its value and (b) its weak correlation with biochemical and immunological parameters of malnutrition. Whereas, the

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limitations of BMI as a biomarker of obesity are related to: (a) an inability to distinguish between fat and fat-free (lean) body mass; (b) a failure to determine fat distribution; (c) a dependence on the accuracy of reported or measured height; and (d) the influence of age, gender, and comorbidities on the accuracy of the cut-offs used in the diagnosis of obesity. Nevertheless, BMI correlates with: (a) central body fat distribution; (b) laboratory biomarkers of metabolic (e.g., blood glucose, lipids, uric acid), inflammatory (e.g., C-reactive protein, interleukin-6, and tumor necrosis factor alpha), and endothelial (e.g., VEGF and ICAM) abnormalities. BMI is also useful as: (c) a risk factor (biomarker) in the development of a number of health conditions, such as diabetes mellitus, hypertension, infectious disease, and psoriasis; (d) as a prognostic factor for all-cause and cardiovascular mortality, in-hospital all-cause mortality, surgery complications and outcomes, hospital-acquired (nosocomial) infections, length of in-hospital stay, and risk of readmission; as well as (e) a biomarker for monitoring the clinical and metabolic effects of interventions on weight reduction, including bariatric surgery. This chapter presents an overview of scientific works related to the use of BMI as a biomarker for various clinical disorders and their clinical course.

Keywords

Body mass index · Obesity · Malnutrition · Nutritional status assessment · Risk factor · Biomarker · Prognosis · Weight Fluctuation · Accuracy · Sensitivity · Specificity

Abbreviations

ABSI	A Body Shape Index
AUC	Area under the curve
BF%	Body fat percentage
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CHF	Chronic heart failure
CI	Confidence interval
CKD	Chronic kidney disease
COLD	Chronic obstructive lung disease
CRP	C-reactive protein
CT	Computed tomography
cTnT	Cardiac troponin T
CVD	Cardiovascular disease
DXA or *DEXA*	Dual-energy X-ray *absorptiometry*
ESPEN	European Society of Parenteral and Enteral Nutrition
FFMI	Fat-free mass index
FMI	Fat mass index
GLIM	Global Leadership Initiative on Malnutrition
HDL	High-density lipoprotein

HR	Hazard ratio
LDL	Low-density lipoprotein
MEONF-II	Minimal Eating Observation and Nutrition Form – Version II
MHO	Metabolically healthy obese
MNA	Mini Nutritional Assessment
MONW	Metabolically obese normal weight
MRI	Magnetic resonance imaging
MST	Malnutrition Screening Tool
MUHO	Metabolically unhealthy obese
MUST	Malnutrition Universal Screening Tool
NASH	Nonalcoholic steatohepatitis
NPV	Negative predictive values
NRS-2002	Nutritional Risk Screening 2002
NSI	Nutritional Screening Initiative
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NUFFE	Nutritional Form For the Elderly
NWO	Normal weight obesity
ONS	Oral nutritional support
OR	Odds ratio
PAD	Peripheral artery disease
PG-SGA	Patient-Generated Subjective Global Assessment
PPV	Positive predictive values
ROC	Receiver operating characteristic curve
WC	Waist circumference
WHO	World Health Organization
WHR	Waist-to-hip ratio
WHtR	Waist-to-height ratio

Introduction

Body mass index (BMI) as a tool for assessing nutritional status, mainly among adults, was first described by Adolphe Quetelet in the mid-nineteenth century (Romero-Corral et al. 2008; Sorkin 2014). This parameter is calculated as a quotient of current body weight in kilograms and the square of the height expressed in meters (Romero-Corral et al. 2008). The Expert Group of the World Health Organization (WHO) recommends the following BMI ranges for White and Black adults over 20 years of age: underweight <18.5 kg/m^2; normal weight 18.5–24.9 kg/m^2; overweight 25–29.9 kg/m^2; and obesity ≥ 30 kg/m^2 (Cosentino et al. 2020; Visseren et al. 2021). For people of Asian/Chinese ethnicity, the proposed BMI ranges have lower cut-off values: underweight <18.5 kg/m^2; normal weight 18.5–22.9 kg/m^2; overweight 23–24.9 kg/m^2; and obesity ≥ 25 kg/m^2 (Table 1). For individuals of Chinese ethnicity, the criteria for BMI ranges are: underweight <18.5 kg/m^2; normal weight 18.5–23.9 kg/m^2; overweight 24–27.9 kg/m^2; and obesity ≥ 28 kg/m^2. However, some authors do not recommend the use of race-specific BMI cut-offs and, instead,

Table 1 Proposed BMI cut-off values for nutritional status assessment

Nutritional condition	WHO: White and Black populations (kg/m^2)	WHO: Asian population (kg/m^2)	Prospective Studies Collaboration/ ESC (kg/m^2)	ESPEN GLIM (age range) (kg/m^2)	ESPEN GLIM: Asian population (age range) (kg/m^2)	Other cut-off values suggested (surveys) (kg/m^2)	Other cut-off values suggested (kg/m^2)
Severe underweight/ malnutrition	<16.49			<18.5 (<70 y) <20 (≥70 y)			
Underweight/ malnutrition	<18.5	<18.5	<20	<20 (<70y) <22 (≥70y)	<18.5 (<70 y) <20 (≥70 y)	<18.5 (NRS-2002, MUST) <19 (MNA-SF) <20 (cachexia/ cancer cachexia) <23 (AKI or CKD)	<17.8 <22.5 <23 <24 <25 <27 (80 y)
Normal weight	18.5–24.9	18.5–22.9	20–25				
Overweight	25–29.9	23–24.9	>25				
Obesity	≥30	≥25 ≥28				>30 (MUST)	≥30 ≥31 ≥33 ≥37
Obesity class I	30–34.9						
Obesity class II	35–39.9						
Obesity class III	≥40						

References based on: Evans et al. 2012; Winter et al. 2014; Tojek et al. 2019; Visseren et al. 2021

Abbreviations: *AKI* acute kidney insufficiency, *BMI* body mass index, *CKD* chronic kidney disease, *ESC* European Society for Cardiology, *ESPEN* European Society of Parenteral and Enteral Nutrition, *GLIM* Global Leadership Initiative on Malnutrition, *MNA-SF* Mini Nutritional Assessment-Short Form, *MUST* Malnutrition Universal Screening Tool, *NRS-2002* Nutritional Risk Screening 2002, *WHO* World Health Organization

age-specific cut-offs for BMI are strongly suggested (Winter et al. 2014; Tojek et al. 2019; Cosentino et al. 2020; Visseren et al. 2021).

A high BMI ($\geq$30 kg/m^2) has been used as a tool for diagnosing obesity in a number of epidemiological and clinical studies and has also been recommended for individual use in clinical practice in therapy focused on weight loss, despite the widely acknowledged limitations of BMI, and, in particular, its undesirably low sensitivity as a marker (<50%). On the other hand, BMI value correlates with anthropometric indices of central fat distribution (e.g., waist circumference [WC], waist-to-hip ratio [WHR], waist-to-height ratio [WHtR], A Body Shape Index [ABSI], and conicity index) and with blood concentration of biomarkers of the risk and prognosis of various, mostly cardiovascular, diseases (e.g., metabolic, immunological, and inflammatory conditions) (Prospective Studies Collaboration 2009; Evans et al. 2012; Streng et al. 2017; Bragança et al. 2020; Suthahar et al. 2021). Moreover, a high BMI, as a single parameter, is recognized as a risk factor for many diseases (e.g., diabetes mellitus, hypertension, dyslipidemia, hyperuricemia, musculo-skeletal diseases, and colorectal cancer). Whereas, a low BMI (<18.5 kg/m^2) is used as a single index of malnutrition or as one of the parameters in surveys of patients' nutritional risk assessment, such as the Nutritional Risk Screening 2002 (NRS-2002) tool, Malnutrition Universal Screening Tool (MUST), Mini Nutritional Assessment (MNA), Nutritional Form For the Elderly (NUFFE), and Minimal Eating Observation and Nutrition Form – Version II (MEONF-II) (van Bokhorst-de van der Schueren et al. 2014; Cederholm et al. 2019). Low BMI (<18.5 kg/m^2) also has negative predictive power for all-cause mortality, prolongation of in-hospital stays, complications after surgery, and risk of readmission (Romero-Corral et al. 2008; Spychalska-Zwolińska et al. 2018; Ammann et al. 2019; Anaszewicz et al. 2019; Wawrzeńczyk et al. 2019; Chiu et al. 2021; Visseren et al. 2021). By contrast, large sample analysis of medical documentation failed to show any associations between BMI and in-hospital all-cause mortality (Budzyński et al. 2016; Tojek et al. 2019).

In light of the above-mentioned discrepancies concerning the clinical usefulness of BMI values, this chapter summarizes the current knowledge of the clinical use of BMI as (a) a biomarker of a patient's nutritional status, (b) BMI-associated risk of disease development, (c) BMI-related out- and inpatient prognoses, and (d) the relationships between BMI value and other anthropometric and body composition measures.

BMI as a Biomarker of Nutritional Status

BMI is still used and recommended as a tool for nutritional risk and status assessment (Nuttall 2015; Visseren et al. 2021), in spite of the introduction into practice of new anthropometric formulas for nutritional status assessment and/or adiposity distribution, such as WHR, WHtR, ABSI, conicity index, and the greater availability of body composition measures (Sommer et al. 2020; Zhu et al. 2020). As stated above (see also Table 1), according to the Expert Group of the WHO, the BMI ranges

widely used for White and Black populations are: underweight <18.5 kg/m^2; normal weight 18.5–24.9 kg/m^2; overweight 25–29.9 kg/m^2; and obesity ≥30 kg/m^2 (Budzyński et al. 2016). However, the Prospective Studies Collaboration (2009) suggests that a BMI range of 22.5–25 kg/m^2 is related to the lowest all-cause mortality, and Nuttall (2015) and the European Society for Cardiology (ESC) group for cardiovascular diseases prevention (Visseren et al. 2021) suggest the use of a BMI range of 20–24.9 kg/m^2 as a criterion for a target (desirable) BMI value that is linked with the lowest all-cause mortality (Cosentino et al. 2020; Visseren et al. 2021). This suggests that BMI values of <18.5, <20, or <22.5 kg/m^2 could be recognized as cut-offs for the diagnosis of underweight or malnutrition, and a BMI between 25 and 30 kg/m^2 (25 ≤ BMI <30 kg/m^2) could be used as a biomarker cut-off for the diagnosis of overweight and/or obesity, not only in individuals of Asian ethnicity, but also in White and Black populations.

BMI as a Biomarker of Malnutrition

Malnutrition is a heterogeneous syndrome caused by an intake and/or absorption of nutrients inadequate to meet daily demand or as "a state resulting from lack of uptake or intake of nutrition leading to decreased lean and body cell mass leading to diminished physical and mental function and impaired clinical outcome from disease" (Cederholm et al. 2015). Low BMI is frequently used as a biomarker of malnutrition or undernutrition, in WHO, Global Leadership Initiative on Malnutrition (GLIM), and European Society of Parenteral and Enteral Nutrition (ESPEN) statements, and in scores for nutritional risk assessment, such as NRS-2002 and MUST (Cederholm et al. 2015; Cederholm et al. 2019). The WHO advocates a BMI of <18.5 kg/m^2 as a general cut-off for underweight. However, the use of this cut-off seems to be justified only among young and healthy populations for the following reasons: (a) patients with unintentional loss of weight of more than 10% in previous 3 months (a widely accepted index of high malnutrition risk, e.g., according to the NRS-2002 survey) may have a BMI in the normal range, and may even be diagnosed as overweight or obese; (b) even severely malnourished patients with chronic hypoalbuminemia may have a high BMI due to fluid retention; (c) BMI value may be maintained among inpatients suffering from severe acute diseases with serious inflammatory response and may even increase, despite the progress of malnutrition, due to fluid retention and the development of generalized edema in the course of capillary leak and quickly progressing hypoalbuminemia, as well as possible progress in cardiac and kidney insufficiency; for the same reasons, BMI does not seem to be suitable as a biomarker for monitoring the outcomes of malnutrition treatment; (d) BMI does not distinguish between lean and fat mass and a decrease in fat-free mass is an indicator of malnutrition; and (e) the accepted predictive values of a normal BMI interval, for example, in the prediction of survival, are different for the general population, i.e., <18.5 kg/m^2, <20 kg/m^2 (Visseren et al. 2021), <22.5 kg/m^2 (Prospective Studies Collaboration 2009), for older people (namely, <20 kg/m^2 for individuals <70 years of age, and <22 kg/m^2 for subjects 70 years

and older) (Cook et al. 2005; Cederholm et al. 2015; Cederholm et al. 2019), and for patients with chronic obstructive lung disease (COLD), in whom a BMI of <21 kg/m^2 is suggested as a cut-off for the recommendation of oral nutritional support (ONS). Moreover, some authors propose age-specific cut-offs for BMI ranges for a diagnosis of underweight, such as: <22 kg/m^2, <23 kg/m^2, <24 kg/m^2, and even <25–27 kg/m^2 for females, <27–29 kg/m^2 for males over the age of 65, and even <27 kg/m^2 for patients over the age of 80 (De Hollander et al. 2012; Winter et al. 2014). Therefore, there is a need to update and generalize the BMI cut-offs for malnutrition diagnosis, particularly among patients older than 65 (Winter et al. 2014; Gonzalez et al. 2017; Tojek et al. 2019).

Two alternative ways to diagnose malnutrition in Europe have been identified in the recent GLIM recommendations (Cederholm et al. 2019) and an ESPEN consensus statement: (a) the presence of a BMI of <18.5 kg/m^2 as a single biomarker; and (b) fulfilment of combined criteria composed of low BMI (or a low fat-free mass index [FFMI]) and the occurrence of an unintentional weight loss of $>10\%$ over an indefinite time period, or $>5\%$ over the previous 3 months, wherein a low BMI value is defined as <20 kg/m^2 if <70 years of age, or <22 kg/m^2 if ≥ 70 years of age; the cut-off value for a low FFMI is considered to be <15 kg/m^2 in women and <17 kg/m^2 in men (Cederholm et al. 2015; Cederholm et al. 2019). In populations of Asian ethnicity, the proposed cut-off values for BMI are lower: <18.5 kg/m^2 for patients of <70 years of age, or <20 kg/m^2 if the patient is 70 years of age or older. Moreover, GLIM recommends that a diagnosis of malnutrition is required to fulfil both a phenotypic criterion (i.e., body weight loss, low BMI or low FFMI) and an etiological criterion for malnutrition (i.e., reduced food intake and an elevated blood C-reactive protein [CRP] concentration of >5 mg/L). Furthermore, GLIM proposes different age-specific BMI cut-offs for the diagnosis of severe malnutrition, which should be diagnosed in patients who have experienced unintentional weight loss either of $>10\%$ within the previous 6 months or $>20\%$ beyond 6 months, and whose current BMI is <18.5 kg/m^2 if the patient is below 70 years of age or <20 kg/m^2 if the patient is at least 70 years of age (Cederholm et al. 2019; Einarsson et al. 2020). A study by Maeda et al. (2020) suggests different optimal BMI cut-off values for a malnutrition diagnosis of <17 kg/m^2 for younger patients (<65 y) and <17.8 kg/m^2 for older patients (≥ 65 y).

Malnutrition is related to prolonged length of in-hospital stay, a reduction in quality of life, delayed wound healing, increased risk of adverse health conditions such as infection and functional capacity, and treatment complications, as well as to a higher risk of morbidity and mortality, especially in the elderly. Therefore, routine screening for malnutrition and/or malnutrition risk using validated surveys is recommended in the majority of developed countries. In a study by Ye et al. (2018), MUST (with BMI <18.5 kg/m^2 as the cut-off for the highest malnutrition score) and MNA (with BMI <19 kg/m^2 as the cut-off for the highest score) had the same sensitivity (94.1%), and NRS-2002 (with BMI <18.5 kg/m^2 as the cut-off for the highest score) had the lowest sensitivity (92.2%) for a malnutrition diagnosis according to the GLIM criteria. Moreover, MUST had the highest specificity (76.5%) for malnutrition diagnosis compared with NRS-2002 (57.8%) and MNA

(63.7%); MUST also had the highest positive predictive value (PPV) (50%) and negative predictive value (NPV) (98.1%) (Ye et al. 2018). However, in a study by Clark et al. (2020), assessing the accuracy of the Malnutrition Screening Tool (MST) (which does not have BMI as a parameter), MST score had a sensitivity of 56.7%, a specificity of 69%, and an area under the curve (AUC) of 0.63 when compared with the GLIM criteria, and a sensitivity of 60.7%, a specificity of 58%, and an AUC of 0.59 when compared with the ESPEN criteria. In a study of 562 cancer patients, for all criteria combinations of GLIM together versus Patient-Generated Subjective Global Assessment (PG-SGA), sensitivity was 60.4% (95% confidence interval [95% CI]: 53.8–66.7), specificity was 97.9% (95% CI: 95.4–99.1), while the accordance between GLIM and PG-SGA was moderate ($\kappa = 0.614$) (Wang et al. 2021). In another study, malnutrition risk was present in 16% of all patients according to MUST and in 42% according to PG-SGA. Among patients with a BMI <25 kg/m^2, MUST identified 31% of the patients as being at nutritional risk, and PG-SGA identified 52%. However, in patients with a BMI ≥ 25 kg/m^2, MUST identified 5% of the patients as being at nutritional risk and PG-SGA 36%. Of the overweight/obese patients at nutritional risk according to PG-SGA, 90% were categorized as low risk by MUST (van Vliet et al. 2021).

In addition to the surveys enumerated above, BMI also revealed some, but mostly weak, correlations with other biomarkers of nutritional status assessment, such as blood concentrations of albumin ($r = 0.144$), plasma lipids (e.g., triglycerides, $r = 0.365$), and total lymphocyte count. Only a few articles were found on the accuracy of BMI in the diagnosis of malnutrition (see Table 2). On the other hand, scores in MUST and MST surveys predicted low BMI or unintentional weight loss with a sensitivity and specificity of 58% and 98% (MUST) and 78% and 94% (MST), respectively (Power et al. 2018).

Overweight and Obesity

Obesity is widely recognized as a pandemic public health problem. Nevertheless, the methods available for diagnosing obesity are still insufficiently specific (Vasconcelos et al. 2010). Overweight and obesity are defined as excessive fat accumulation that may impair health. Usually, obesity is diagnosed when body fat percentage (BF%) exceeds 25–30% in males, and 30–35% in females (Vasconcelos et al. 2010). During the last 30 years, a BMI of ≥ 30 kg/m^2 as proposed by the WHO has been used as a biomarker of obesity. BMI is easy and cheap to obtain; however, several researchers and professional associations consider the use of BMI as the biomarker of obesity insufficient, when taking into account: (a) the absence of a "gold standard" for diagnosing obesity (Romero-Corral et al. 2008; Ross et al. 2020; Sommer et al. 2020; Zhu et al. 2020); (b) the heterogenous character of obesity (various causes [i.e., primary and secondary], different fat distributions [e.g., android and gynoid], and diverse metabolic and clinical consequences [e.g., diabetes mellitus and nutrition-related cancer]); and (c) the several limitations of BMI, of which the most important are as follows: (a) an inability to distinguish between fat and fat-free

Table 2 Diagnostic utility of BMI as a biomarker of malnutrition

No.	Author(s)	Study design/ reference method (gold standard)	Number of patients	Outcome/effect measure/BMI (kg/m^2)	BMI cut-off (kg/m^2)
1.	Campillo et al. (2004)	Prospective study/ severe malnutrition defined as mid-arm muscular circumference and triceps skinfold < 5th percentile of a reference population when aged ≤74 y or the 10th percentile when aged ≥75 y	1052	*BMI ≤ 20: sensitivity in detecting severe malnutrition*: In patients after surgery on a hip fracture: 100% In patients undergoing palliative care for cancer: 80% In elderly patients with medical affections: 100% In patients with liver cirrhosis and tense ascites: 40% In patients with cardiac disease: 33.3% In patients with stroke: 50%	<20
2.	Hassanin et al. (2021)	Single-centre cross-sectional study/malnutrition diagnosis according to PG-SGA	98 haemodialysed patients; mean age: 51.3 y	*BMI range sensitivity in detecting malnutrition*: BMI <18.5: 2%; BMI <23: 17.3%; BMI <23: correlated with serum albumin ($r = 0.224$; $p = 0.028$) BMI cut-offs (<23 kg/m^2 or <18.5 kg/m^2) did not show any agreement with diagnosed malnutrition, but a DMS ≥14 showed a sensitivity of 84.3% and a specificity of 60.7%	<18.5 <23
3.	Maeda et al. (2020)	Single-centre, cross-sectional/ GLIM-defined malnutrition	6783 patients aged ≥40 y Asian population	BMI <18.5: sensitivity 71.4%, specificity 56% Diagnosis of inflammatory disease: sensitivity 66.8%, specificity 63%	<17 for patients aged 40–69 y <17.8 for patients aged ≥70 y

(continued)

Table 2 (continued)

No.	Author(s)	Study design/ reference method (gold standard)	Number of patients	Outcome/effect measure/BMI (kg/m^2)	BMI cut-off (kg/m^2)
4.	Ng et al. (2019)	Retrospective study/malnutrition according to SGA	1152 Age ≥65 y	*BMI cut-offs in detecting malnutrition (SGA)*: BMI <26: sensitivity 80.8%, specificity 61.5%; AUC of the ROC curve analysis for BMI: 0.802, 95% CI: 0.773–0.830; $p < 0.0001$ BMI <23: had the highest agreement ($k = 0.458$) with SGA score BMI <18.5–23: no significant utility in relation to malnutrition diagnosis	<26
5.	Thomas et al. (2002)	Prospective study/ MNA score <17	837 at a subacute-care centre, age: 76 ± 13 y	BMI <19: sensitivity 41%, specificity 86% BMI <22: sensitivity 70%, specificity 71% No relation between BMI and length of stay and mortality	<21
6.	van Vliet et al. (2021)	Observational cohort study/ MUST (≥2) and PG-SGA SF score (≥9) for malnutrition risk	430 aged ≥18 y, overweight/ obese inpatients	*Malnutrition risk assessed by MUST score (≥2)*: BMI718.5: 100%; BMI 18.5–24.9: 8.7%; BMI ≥25: 2.7% BMI ≥30: 3.7% *Malnutrition risk assessed by PG-SGA SF score (≥9)*: BMI <18.5: 91%; BMI 18.5–24.9: 22.4%; BMI ≥25: 12.7% BMI ≥30: 19.4% As many as 20% of obese patients may be malnourished based on PG-SGA score	<25 ≥25

Abbreviations: AUC area under the curve, BMI body mass index, DMS Dialysis Malnutrition Score, GLIM Global Leadership Initiative on Malnutrition, MNA Mini Nutritional Assessment, MUST Malnutrition Universal Screening Tool, PG-SGA SF Patient-Generated Subjective Global Assessment-Short Form, ROC receiver operating characteristic, SGA Subjective Global Assessment

(lean) body mass, which may be a cause of obesity underestimation in individuals of normal weight with a high percentage of adipose tissue (normal weight obesity), and overestimation of overweight or obesity in those with a muscular body build (e.g., athletes) or with edema (e.g., in severely malnourished individuals); (b) an inability to determine fat distribution (android or gynoid), despite BMI correlating with WC, WHR, WHtR, and conicity index (Evans et al. 2012); (c) the dependence of BMI on the accuracy of measuring height, which frequently decreases with a patient's age and causes problems with interpreting BMI changes over the course of a life; (d) the influence on BMI of: (d1) age (e.g., the prognostic power of different BMI ranges depending on whether patients are <65 or ≥65 years); (d2) gender (women generally have a higher BF%); (d3) race (e.g., different BMI ranges for White/Black, Asian, and Chinese ethnicities); (d4) smoking status (e.g., smoking cessation frequently leads to an increase in BMI, despite the patient being in the normal weight range during the majority of their life, which may lead to a misdiagnosis of the risk of nutrition-related diseases); and (d5) co-morbidities affecting body weight (e.g., bowel resection and artificial joint implantation), and/or co-morbidities associated with a decrease in the patient's height (e.g., due to loss of limb segments in accidents or in the course of peripheral artery disease [PAD], senile kyphosis, spinal vertebra compression in the course of osteoporosis, or postural changes, secondary sarcopenia and the loss of muscle tone) (Cook et al. 2005; Antonopoulos et al. 2016; Budzyński et al. 2016; Tojek et al. 2019). The "c" and "d5" limitations concerning a patient's height should also be taken into account with regard to WHtR value.

Despite the limitations of BMI enumerated above, a BMI between 25 and 30 kg/m^2 (25 ≤ BMI <30 kg/m^2) is still used as a biomarker of overweight and/or obesity. Therefore, old epidemiological data concerning, for example, obesity prevalence based on BMI value alone, should be interpreted with care. Such a statement is justified in the light of data showing the very low sensitivity of BMI ≥30 kg/m^2 as a cut-off in comparison with the widely accepted gold standard for obesity diagnosis, high BF%, irrespective of the BF% examination used (e.g., dual-energy X-ray *absorptiometry* [*DXA* or *DEXA]*, bioelectrical impedance analysis [BIA], computed tomography [CT], or magnetic resonance imaging [MRI]). In comparison with the suggested cut-offs for increased BF% (e.g., >25% for men, and >30% for women), the sensitivity of BMI ≥30 kg/m^2 for obesity diagnosis amounts to 51.4–62.4% in women and 34–57% in men, and the specificity to 88.1–99% in women and 98% in men (Romero-Corral et al. 2008; Sommer et al. 2020) (see Table 3). Similarly, in several studies performed in the United States and Canada, BMI ≥30 kg/m^2 had a high PPV (66–93%) but low sensitivity (8–30%) (Peng et al. 2017; Ammann et al. 2019). This means that only 30–62.4%, and even 8% of obese individuals, who were recognized as obese based on the gold standard, were also classified as obese according to BMI, and 1–11.9% of individuals, who were non-obese according to the gold standard, were identified as obese based on BMI (Samadi et al. 2013). Vasconcelos et al. (2010) compared the accuracy of two BMI cut-off points promoted by the WHO (BMI ≥25 and ≥30 kg/m^2) and the Nutritional Screening Initiative (NSI) (BMI ≥27 kg/m^2) in relation to BF% determined in DXA as a

Table 3 Diagnostic utility of BMI as a biomarker of obesity

No.	Author(s)	Study design/reference method/gold standard for obesity diagnosis	Number of patients	Outcome/effect measure: BMI (kg/m^2)	BMI cut-off value (kg/m^2)
1.	Alammar et al. (2020)	Retrospective cross- sectional study/BF % measured by DEXA (>25% for men, >35% for women)	941 Saudi Arabian subjects (348 men, 593 women)	*BMI ≥30 kg/m^2 sensitivity and specificity vs. DEXA* Men: 34% (95% CI: 29–40) and 98% (95% CI: 90–99) Women: 55% (95% CI: 51–59) and 93% (95% CI: 69–99)	>30
2.	Banack et al. (2018)	Retrospective study/BF% measured by DEXA (three ranges: ≥35%, ≥38%, and ≥40%)	1329 post- menopausal women	*BMI ≥30 kg/m^2 sensitivity and specificity vs. BF%* Sensitivity: 32.4%, 44.6%, 55.2%, respectively Specificity: 99.3%, 97.1%, 94.6%, respectively BMI cut-off in ROC analysis: 24.9 kg/m^2 for 35% BF; 26.49 for 38% BF, and 27.05 for 40% BF	≥30 ≥24.9 ≥26.5 ≥27.05
3.	Okorodudu et al. (2010)	Systematic review and meta-analysis of 25 articles/BF% measured by DEXA, BIA, ADP, HW, ID, SF	31,968 adults	*BMI ≥30 kg/m^2 sensitivity and specificity vs. BF%* Pooled BMI ranges: 50% (95% CI: 43–57) and 90% (95% CI: 86–94) BMI ≥30 kg/m^2: 42% (95% CI: 31–43) and 97% (95% CI: 96–97)	≤24.9 25–30 ≥30
4.	Romero-Corral et al. (2008)	Retrospective cross-sectional study/BF % measured by BIA (>25% for men, >35% for women)	13,601 (48% men), US	*BMI ≥30 kg/m^2 sensitivity and specificity vs. BIA* Men: 36% and 95% Women: 49% and 99% BMI correlated with BF% in men ($r = 0.44$) and women ($r = 0.71$)	>30

5.	Sommer et al. (2020)	Meta-analysis of 36 articles/BF% measured by DEXA, BIA, MRI, CT	25,328 (14,008 women and 11,320 men)	*BMI ≥30 kg/m^2 sensitivity and specificity vs. BF%* Men: 49.6% (95% CI: 34.8–64.5) and 97.3% (95% CI: 92.1–99.1) Women: 51.4% (95% CI: 38.5–64.2) and 95.4% (95% CI: 90.7–97.8)	Men: 23.5–30 Women: 19.6–30 ≥30 in 75% of studies
6.	Vasconcelos et al. (2010)	Cross-sectional; BF% measured by DEXA	180 elderly subjects (60 men and 120 women)	*BMI ≥30 sensitivity and specificity vs. DEXA* Men: BMI ≥27.58: 73.7% (95% CI: 48.8–90.8) and 85% (95% CI: 70.2–94.3) Women: BMI ≥22.89: 88.6% (95% CI: 81.3–93.8) and 100% (95% CI: 48–100)	≥27.58 (men) ≥22.89 (women)
7.	Zhu et al. (2020)	Prospective cross-sectional study/BF% measured by BIA (≥27% for obesity and ≥22% for overweight)	353 Chinese military personnel and 380 age-matched control	*BMI ≥28 kg/m^2 sensitivity and specificity vs. BF% – 17.7%, and 80.8%* BMI overestimated the prevalence of obesity and overweight for Chinese military personnel (due to high levels of muscle mass and fat-free mass)	Obesity: ≥28 Overweight: 24–28

Abbreviations: *ADP* air-displacement plethysmography, *BF%* body fat percentage, *BIA* bioelectrical impedance analysis, *BMI* body mass index, *CI* confidence interval, *CT* computed tomography, *DEXA* dual-energy X-ray absorptiometry, *HW* hydrostatic weighing, *ID* isotope dilution, *MRI* magnetic resonance imaging, *SF* skinfold

gold standard for obesity diagnosis in elderly people (≥60y). These authors found greater sensitivity and specificity in the NSI than the WHO cut-offs for elderly men (73.7% and 72.5%, respectively), whereas, among women aged ≥65y, BMI ≥25 kg/m^2 (the WHO criterion for overweight) achieved the greatest sensitivity (76.3%) and specificity (100%), indicating a difference in the most accurate cut-off for diagnosing obesity in elderly women and men. In this study, BMI ≥30 kg/m^2 offered very low sensitivity (28.9%) among women (Vasconcelos et al. 2010). A subgroup analysis that excluded studies on Asian men and focused on men of White, Latin, or mixed ethnicity (6 studies, n = 5991, cuts-offs: 28.5–30 kg/m2) showed little effect: a sensitivity of 52.8% (95% CI: 36.4–68.6) and a specificity of 98.9% (95% CI: 93.8–99.8).

In another study, the sensitivity of BMI ≥30 kg/m^2 for obesity diagnosis (BF% in DXA) was only 55% (95% CI: 51–59) and 34% (95% CI: 29–40) and specificity amounted to 93% (95% CI: 69–99) and 98% (95% CI: 90–99) in women and in men, respectively (Alammar et al. 2020). However, when these authors used ROC curve analysis, the most appropriate BMI cut-off value for the diagnosis of obesity was established at 24 kg/m^2 and the sensitivity of this BMI cut-off was higher: 91% (95% CI: 88–93) for females and 85% (95% CI: 80–88) for males (Alammar et al. 2020). In a study involving 1329 post-menopausal women participating in the Buffalo OsteoPerio Study, compared with BF% determined in DXA at the values of 35%, 38%, and 40% as a gold standard, the sensitivity of BMI ≥30 kg/m^2 amounted to 32.4%, 44.6%, and 55.2%, respectively. Using ROC curve analysis, the authors established the following new cut-off points for BMI: 24.9 kg/m^2 for 35% BF%, 26.5 kg/m^2 for 38% BF%, and 27.1 kg/m^2 for 40% BF% (Banack et al. 2018). Prior to this, Winter et al. (2014) had proposed BMI ≥33 kg/m^2 as a cut-off for the diagnosis of obesity in elderly patients. In another study, looking at obesity classification in children, compared with a fat mass index (FMI), the BMI cut-off of ≥21.2 kg/m^2 had a sensitivity of 79% and a specificity of 73% (Samadi et al. 2013).

In light of the limitations of BMI enumerated above, including the low sensitivity, updating the classic ranges of BMI values established by the WHO has been postulated in recent years, and the validation of new, cheap, and safe methods for obesity diagnosis are required.

BMI as a Biomarker of Fat Distribution and Body Composition

Despite the limitations mentioned in respect of BMI for the diagnosis of malnutrition and obesity, some studies have pointed out moderate correlations (r = 0.5–0.7) between BMI value and BF%. The strongest example of this association is the following formula for BF% calculation on the basis of BMI: BF% = 1.2 × BMI + 0.23 × age − 10.8 × gender (1 for males, 0 for females) − 0.54; however, this formula is only applicable to young and healthy adults (Tojek et al. 2019). A hypothesis has also been formulated that the accuracy of BMI for obesity diagnosis could be improved if two or more anthropometric or body composition parameters of obesity were taken together and, therefore, the relationships between BMI and

parameters of adiposity distribution were analyzed. For example, pooled analysis of WC had a sensitivity and specificity of 50% (95% CI: 43–57) and 90% (95% CI: 86–94), and BMI $\geq$30 kg/m^2 specificity was 95.4% and 97.3% for the diagnosis of high BF% in women and men, respectively (Okorodudu et al. 2010). In a study by Sommer et al. (2020) of BMI, WC, WHR, and WHtR, BMI $\geq$30 kg/m^2 had the lowest sensitivity in detecting obesity, which amounted to 51.4% for women (95% CI: 38.5–64.2) and 49.6% for men (95% CI: 34.8–64.5). This BMI cut-off had a specificity of 95.4% (95% CI: 90.7–97.8) in women and 97.3% (95% CI: 92.1–99.1) in men. However, the authors emphasize that, due to the lack of other simple tools, BMI and WC can be used in the initial phase of obesity diagnosis (Sommer et al. 2020). A study by Evans et al. (2012) showed that BMI correlated with other anthropometric parameters of nutritional status assessment; however, higher BMI was more weakly associated with an increased risk of chronic kidney disease (CKD) and cardiovascular disease (CVD) than higher WC and conicity index (Evans et al. 2012). Similarly, in a study by Gadekar et al. (2018), BMI correlated with parameters of abdominal fat distribution in men ($r = 0.76$) and in women ($r = 0.61$); however, in the diagnosis of central adiposity, the sensitivity and specificity of BMI were lower (78.8% and 85.4% for men, and 57.1% and 79.3% for women) than for WHR (90.9% and 98.9% for men, and 96.4% and 79.3% for women). Studies in adolescents have shown that BMI and BF% only have a strong correlation for the highest percentiles of BMI, while in the lower percentiles, the correlation was weak, with the result that more than half of the subjects with increased BF% were misdiagnosed as normal or overweight using BMI. This misclassification was not corrected by lowering the cut-off value for obesity to $\geq$25 kg/m^2, which was still associated with the misclassification of 38% of overweight males and 16% of overweight females as non-obese (Romero-Corral et al. 2008). However, in a study by Gasier et al. (2015), strong relationships between BMI and BF% measured by DXA ($r > 0.7$) and between BMI and BF% and lean mass measured by BIA ($r \sim 0.7$) were found. It should be underlined that in every obese patient, especially the elderly, the dietician and physician ought to be aware of the risk of sarcopenic obesity or malnutrition, such as in a course of treatment for weight reduction, or due to social problems and food insecurity.

BMI Applications to Other Diseases or Conditions

Obesity and abdominal fat distribution are the main criteria for metabolic syndrome diagnosis (Streng et al. 2017; Visseren et al. 2021). Higher BMI is also recognized as a risk factor for many chronic diseases, such as type 2 diabetes mellitus, diabetic retinopathy, cardiovascular diseases (e.g., coronary artery disease, PAD [hazard ratio (HR): 1.09–2.0], atrial fibrillation [HR: 1.34–4.51], chronic cardiac failure [HR: 1.16–1.41]), ischemic stroke, with a 4% risk increase for each unit increase in BMI, obstructive sleep apnea, nonalcoholic steatohepatitis (NASH), psoriasis, severity of depressive symptoms in women of child-bearing age, prostate volume in benign prostatic hyperplasia, and the progression of CKD and cancer prevalence

(e.g., colorectal cancer) (Evans et al. 2012; Anaszewicz and Budzyński 2017; Spychalska-Zwolińska et al. 2018; Wawrzeńczyk et al. 2019; Chiu et al. 2021; Kim et al. 2021; Li et al. 2021) (Table 4). In comparison, low BMI is related to a higher prevalence of lung disease, tuberculosis, sarcopenia, and frailty (Tanaka et al. 2021; Valdiviesso et al. 2021), and maintaining BMI during hospitalization for patients with stroke and overweight and obesity was associated with an improvement in scores of activities of daily living after rehabilitation (Kokura and Nishioka 2021).

Positive and negative correlations have also been found between BMI and blood level of the indices of cardiovascular risk (Mkuu et al. 2021). Associations have been shown to be strongest between obesity and the incidence of diabetes mellitus, particularly in women (risk ratio 12.41, 95% CI: 9.03–17.06) (Vasconcelos et al. 2010; Evans et al. 2012; Sommer et al. 2020). Whereas, in a study by Gasier et al. (2015), hypertension was the most commonly reported obesity-related condition (14%), followed by hyperlipidemia (8%), and type 2 diabetes mellitus (4%). Chaudhary et al. (2019) observed that WC and WHR exerted a stronger correlation with blood pressure than BMI; however, among Chinese school-aged children with obesity, BMI exerted greater diagnostic utility in identifying dyslipidemia than other anthropometric measures, although a combination of BMI, WC, and WHR yielded the strongest relationships with individuals with one of the lipid disorders (Zhu et al. 2020). Furthermore, abdominal adiposity contributed to adverse high-density lipoprotein (HDL) cholesterol and triglyceride levels (Zhu et al. 2016). Moreover, in a cross-sectional analysis of 27,158 apparently healthy US women (mean age 54.7 y) participating in a Women's Health Study, BMI ranges according to WHO recommendations were significantly and positively associated with blood concentrations of numerous cardiovascular risk biomarkers, such as CRP, apolipoprotein B100, apolipoprotein A, total and low-density lipoprotein (LDL) cholesterol, fibrinogen, intercellular adhesion molecule type 1, and negatively with HDL cholesterol (Mora et al. 2006). In a study by Bragança et al. (2020), individuals with high BF% also had higher blood concentrations of proinflammatory cytokines, such as interleukin-6 and tumor necrosis factor alpha, which are associated with the risk of atherosclerosis progression, and insulin resistance. However, these unfavorable associations between BMI and levels of other cardiovascular biomarkers are influenced by physical inactivity (Mora et al. 2006). For example, compared with an active woman of normal weight, the odds ratios (ORs) and 95% CIs for CRP >3 mg/L were as follows: for inactive women of normal weight 1.26 (1.15–1.37); for active overweight women 2.68 (2.41–2.98); for inactive overweight women 3.11 (2.84–3.41); for active obese women 8.25 (7.15–9.51); and for inactive obese women 9.86 (8.84–10.99) (Mora et al. 2006). These observations show a strong pro-inflammatory effect of obesity that may be only slightly reduced by higher physical activity (Visseren et al. 2021).

Based on BMI range, BF% and distribution, and on the metabolic consequences of obesity mentioned above, the following basic phenotypes of obesity are enumerated, which may undermine the use of BMI discussed above as a biomarker of metabolic abnormalities and increased cardiovascular risk (De Lorenzo et al. 2016; Gonzalez et al. 2017). These phenotypes are as follows: (a) normal weight obesity (NWO), which includes individuals with a normal range BMI (18.5–24.9 kg/m^2) and

Table 4 BMI as a biomarker of selected diseases

No.	Author(s)	Study design	Number of patients	Follow-up	End-point	Outcome/effect measure
1	Batai et al. (2021)	Retrospective cross-sectional study	278 undergoing surgery for BPO/BPH	–	Pre-operative prostate volume	BMI correlated with prostate volume $r = 0.123$, $p = 0.045$
2	Chang et al. (2021)	Post-hoc analysis of a multicentre, prospective, randomized controlled trial	1000 with CHB age >18 y, undergoing antiviral treatment	72 weeks	NASH resolution	In NASH pts.: BMI ≥23 kg.m^2: OR; 95% CI: 0.41; 0.19–0.90 In non-NASH pts.: BMI ≥23 kg.m^2: OR; 95% CI: 12.51; 2.81–55.61; $p = 0.001$
3		Prospective observational cohort study Cooper Center Longitudinal Study	44,674 men without a history of CVD, BMI <18.5 kg/m^2, mean age 43.4 ± 9.2 y	Mean 19.8 ± 10.4 y	HF mortality	HR; 95% CI; fit vs. unfit, BMI >25 compared with normal weight (p for trend <0.001): BMI 18.5–24.9: 1 vs. 3.96 (2.1–9.6) BMI 25–29.9: 1.72 (1.0–2.9) vs. 3.64 (2.1–6.2) BMI ≥30: 4.47 (2.1–9.4) vs. 6.11 (3.5–10.7)
4	Norden et al. (2021)	Retrospective cohort analysis	>1.5 million from multi-health system and research platform	11 y	Incidence of psoriasis	BMI <25 vs. BMI 25–29.9: aHR; 95% CI: 1.19; 1.12–1.27; $p < 0.001$ BMI 30–34.9: aHR; 95% CI: 1.43; 1.34–1.53; $p < 0.001$ BMI ≥35: aHR; 95% CI: 1.83; 1.71–1.95; $p < 0.001$

(continued)

Table 4 (continued)

No.	Author(s)	Study design	Number of patients	Follow-up	End-point	Outcome/effect measure
5	Nowak et al. (2021)	Multicentre, randomized, double-blind, placebo-controlled trial	1321 with early- stage autosomal dominant polycystic kidney disease	5 y	% change in TKV in MRI	Percentage change in TKV ≥7% vs. <5%; BMI 25–29.9: OR; 95% CI: 2.02; 1.15–3.56 BMI ≥30: OR; 95% CI: 3.76; 1.81–7.80
6	Yang et al. (2021)	Retrospective study	21,647 with DM	–	DR diagnosis	BMI ≥30: aOR; 95% CI: 1.051; 1.048–1.055

Abbreviations: *aHR* adjusted hazard ratio, *aOR* adjusted odds ratio, *BMI* body mass index, *BPO/BPH* benign prostatic obstruction/benign prostatic hyperplasia, *CHB* chronic hepatitis B, *CI* confidence interval, *CVD* cardiovascular disease, *DM* diabetes mellitus, *DR* diabetic retinopathy, *HF* heart failure, *HR* hazard ratio, *MRI* magnetic resonance imaging, *NASH* nonalcoholic steatohepatitis, *OR* odds ratio, *TKV* total kidney volume

BF% >30%; (b) metabolically obese normal weight (MONW) or "thin outside fat inside," which includes individuals with normal range BMI and BF% but with abdominal distribution of adipose tissue, expressed by higher WC, WHR, WHtR, conicity index, and ABSI, and with obesity-related metabolic disorders, such as dyslipidemia, diabetes mellitus, and hyperuricemia; (c) metabolically healthy obese (MHO), represented by individuals with high BMI ($\geq$30 kg/m^2) and BF% >30%, but without the metabolic abnormalities mentioned above; (d) metabolically unhealthy obese (MUHO), defined as individuals with BMI $\geq$30 kg/m^2, BF% >30%, and concomitant metabolic disorders (e.g., diabetes mellitus and dyslipidemia); and (e) individuals with sarcopenic obesity, defined as having BMI $\geq$30 kg/m^2, BF% >30%, and decreased skeletal muscle mass and strength. In one study, the incidence of MHO among obese people was 28.53% and the incidence of MONW among people of normal weight was 30.04% (Wang et al. 2015). In a study by Serrano et al. (2010), adolescents with normal weight obesity and excess weight with high BF% had a worse lipid profile than their normal weight counterparts.

As well as metabolic consequences, higher BMI is also recognized as a risk factor for non-metabolic diseases, such as chronic heart failure (CHF), and is associated with CHF biomarkers (Wawrzeńczyk et al. 2019; Suthahar et al. 2021). In linear regression models, after accounting for potential confounders, BMI positively correlated with cardiac troponin T (cTnT), CRP, procalcitonin, galectin-3, Plasminogen Activator Inhibitor type 1 (PAI-1), mid-regional pro-adrenomedullin, copeptin, and cystatin-C; whereas BMI negatively correlated with N-terminal pro-B-type natriuretic peptide (NT-proBNP), mid-regional pro-A-type natriuretic peptide, C-terminal pro--endothelin-1, and aldosterone. These biomarkers had similar predictive value for incident CHF across the BMI spectrum, and a combination of NT-proBNP and cTnT improved the prediction of heart failure in overweight and obese individuals. Similar observations concerning biomarkers of CHF enabled Streng et al. (2017), in a multifactorial regression analysis based on a population of 2033 in the PROTECT study, to find negative correlations between BMI and NT-proBNP and the receptor for advanced glycation end products, whereas correlations were found between higher BMI and higher levels of uric acid, proadrenomedullin, creatinine, sodium, and bicarbonate. The findings mentioned provide at least the following clinical implications: (a) that relationships between BMI and the risk of the diseases mentioned are U- or J-shaped; (b) that control of a lot of cardiovascular risk factors (e.g., hypertension, dyslipidemia, and insulin resistance) may be achieved by maintenance of normal BMI (Mora et al. 2006); and (c) that the blood concentrations of the enumerated biomarkers (e.g., NT-proBNP) should be interpreted with caution in patients with obesity (Streng et al. 2017).

BMI Applications to Prognosis

As stated above, BMI can be recognized not only as a biomarker of nutritional status and risk, as a risk factor for the development of various diseases, and as an indicator of potential metabolic, inflammatory, and endothelial alterations, but also as a

biomarker of patients' prognoses (Global BMI Mortality Collaboration 2016). Recent data showed a positive linear association between BMI and all-cause mortality (Table 5), which was the lowest in the BMI ranges of 20–25 kg/m^2 among White adults and 22.6–27.5 kg/m^2 among adults of Asian ethnicity (Visseren et al. 2021). These data only partially corroborate the outcomes of older studies, which showed U- or J-shaped associations between BMI and all-cause mortality, with the lowest occurrence (HR: 0.41–0.96) not among patients with the normal weight BMI range mentioned above, but in those with overweight and Class I obesity (BMI 30–34.9 kg/m^2), and even in a higher BMI range (BMI ≥34–37 kg/m^2) in subjects over the age of 65 (Winter et al. 2014; Tojek et al. 2019; Visseren et al. 2021), although not all authors corroborate these data (Sorkin 2014). The association mentioned between higher BMI and lower all-cause mortality is known as the "obesity paradox" or BMI paradox, when the BMI limitations in the assessment of patients' nutritional status enumerated above are taken into account (De Hollander et al. 2012; Sorkin 2014; Antonopoulos et al. 2016; Tojek et al. 2019; Donini et al. 2020; Javed et al. 2020). However, in retrospective analyses of a large sample of inpatients, the existence of any relationships between in-hospital all-cause mortality and BMI range or BMI percentile were not confirmed (Budzyński et al. 2016), despite such an association being found for percentiles of the actual-to-ideal body weight ratio (Tojek et al. 2019).

Low BMI (<18.5 kg/m^2), representing the left part of U- or J-shaped BMI associations with all-cause mortality and as one of the malnutrition criteria, is recognized as a risk factor for mortality in the majority of diseases, as well as a risk factor for complications, readmission, and prolonged stays in patients requiring hospitalization on surgical and medically treated wards (Cederholm et al. 2015; Renfro et al. 2016; Ng et al. 2019; Manne-Goehler et al. 2020; Chiu et al. 2021; Du et al. 2021). In a study by Rondel et al. (2018), malnourished patients had significantly lower 1-year survival rates than those who were well-nourished at 3 months (84% vs. 94%) and 1 year (76% vs. 87%). After adjustments, malnutrition remained significantly and negatively associated with 3-month survival for the Dutch definition for malnutrition (HR for death: 2.25) and the ESPEN diagnostic criteria for malnutrition (HR for death: 2.76). In the Geisinger Rural Aging Study involving 5993 participants aged above 74, compared with individuals with BMI in the normal range, those with BMI <18.5 kg/m^2 had significantly higher all-cause mortality (HR for death; 95% CI: 1.85; 1.09–3.14), and those with BMI of 25–29.9 kg/m^2 had significant lower all-cause mortality rates (HR for death: 0.71; 95% CI: 0.55–0.91) (Ford et al. 2014). In another study, patients with GLIM-defined malnutrition showed significantly higher in-hospital mortality compared with those without malnutrition (Maeda et al. 2020), and low BMI was a predictor of increased mortality and prolonged length of stay after total joint arthroplasty (Kaushal et al. 2021), and increased mortality in patients hospitalized due to non-tuberculous mycobacterial pulmonary disease (Tanaka et al. 2021) and in patients with sepsis (Sato et al. 2021). However, the prognostic power of low BMI, including cut-offs based on WHO recommendations, age-adjusted BMI ranges, and BMI ranges obtained in ROC analysis, was not confirmed in general or in disease-specific patient

Table 5 Impact of BMI on all-cause and cause-specific mortality

No.	Author(s)	BMI cut-off values (kg/m^2)	Study design/reference method (gold standard)	Number of patients	Follow-up	End-point	Outcome/effect measure BMI (kg/m^2)
1.	Bae et al. (2021)	≥18.5 <25	Retrospective observational study, visceral fat area in CT	3661 aged ≥20 y, east Asian patients undergoing cardiovascular surgery	4.6 y (max. 12.2 y)	All-cause and cardiovascular mortality	Normal BMI and visceral fat area: no relation with mortality All-cause mortality vs. subcutaneous fat area: aHR; 95% CI: 0.997; 0.994–1.000 CVD mortality vs. subcutaneous fat area: aHR; 95% CI: 0.994; 0.989–0.999
2.	De Hollander et al. (2012)	<21.1 >31.4	Prospective study	1980 Aged 70–75 y	9–10 y	All-cause and cause-specific mortality; other causes = non-CVD, non-cancer, non-respiratory	BMI range: no association BMI as a continuous variable: all-cause mortality (p for trend <0.01) BMI <20 vs. death from other causes: HR; 95% CI: 2.75; 1.00–7.52 BMI ≥30 CVD mortality: HR; 95% CI: 1.39; 1.00–1.92 All-cause mortality risk: the lowest at BMI 27.1 (24.1–29.3), increased for 21.1< BMI >31.4 CVD mortality risk: the lowest at 25.6 (17.1–28.4), increased for BMI >30.9

(continued)

Table 5 (continued)

No.	Author(s)	BMI cut-off values (kg/m^2)	Study design/reference method (gold standard)	Number of patients	Follow-up	End-point	Outcome/effect measure BMI (kg/m^2)
3.	Du et al. (2021)	≥30 <30	Meta-analysis	109,881 Aged 32–70.5 y	–	COVID-19- specific mortality	Patients aged >60 y: OR; 95% CI: 3.93; 2.18–7.09 BMI >35 kg/m^2: OR; 95% CI: 3.54; 1.48–8.48 For each 1 kg/m^2 increase in BMI: OR; 95% CI: 1.06; 1.02–1.10, $p = 0.002$ For each 2 kg/m^2 increase in BMI: OR; 95% CI: 1.12; 1.04–1.21, $p = 0.002$
4.	García-Ptacek et al. (2014)	<18.5, 25, 30, and 22 "Slim": 18.5–22.9 Normal: 23–24.9; 24.9; 29.9	Cohort study based on SveDem, the Swedish Quality Dementia Registry; 2008–2011; aged 78.5 ± 7.9 y	11,398 with incident dementia	Average 2.5 y	All-cause mortality	BMI <18.5: HR; 95% CI: 1.60; 1.39–1.84 BMI 22–24.9: HR; 95% CI: 0.81; 0.73–0.89 BMI 25–29.9: HR; 95% CI: 0.73; 0.66–0.85 BMI >30 HR; 95% CI: 0.68; 0.59–0.78 BMI <22: each point increase in BMI was associated with a decrease in mortality risk of 11% BMI range 22–25: each point increase in BMI was associated with a decrease in mortality risk of 5% BMI range 25–30: each point increase in BMI was

							associated with a decrease in mortality risk of 3%; decrease in mortality with an increase in BMI up to BMI = 29.9 (men) and BMI = 24.9 (women): obesity paradox
5.		<18.5	Retrospective study	4802 with TJA	Min. 2 y	2-year all-cause mortality or reoperation, 90-day readmission, or extended LOS	BMI <18.5 vs. all-cause mortality: OR; 95% CI: 8.77; 2.14–32.0
6.	Kvamme et al. (2012)	≥25 <25	Retrospective study: Tromsø and HUNT studies Aged >65 y	19,515 (7604 men, 9107 women)	Mean 9.3 y	All-cause and cause-specific mortality	Compared with BMI range 25–27.4 (HR multivariable adjusted; 95% CI): Men: BMI <18.5: aHR; 95% CI: 2.32; 1.75–3.07 Men BMI ≥30: aHR; 95% CI: 1.53; 1.21–1.95 Women: BMI <18.5: aHR; 95% CI: 1.78; 1.37–2.31 Women: BMI ≥30: aHR; 95% CI: 1.45; 1.25–1.67 Men: BMI range 25–29.9 the lowest mortality in men Women: BMI range 25–32.4 had the lowest mortality in women BMI as a continuous variable: a 20% increase in mortality per 2.5 kg/m^2 decrease in BMI in the BMI range <25

(continued)

Table 5 (continued)

No.	Author(s)	BMI cut-off values (kg/m^2)	Study design/reference method (gold standard)	Number of patients	Follow-up	End-point	Outcome/effect measure BMI (kg/m^2)
							BMI as a continuous variable: a 7–9% increase in mortality per 2.5 kg/m^2 decrease in BMI in the BMI range ≥25 BMI as a continuous variable: a 54–74% increase in mortality from respiratory diseases per 2.5 kg/m^2 decrease in BMI in the BMI range <25 U-shaped relationships between WC and all-cause mortality
7.	Manne-Goehler et al. (2020)	<18.5, 18.5–24.9, 25–29.9, 30–34.9, ≥35	Prospective study, South Africa	9728 aged >30		All-cause and cause-specific mortality	BMI range vs. 18.5–24.9: BMI ≤ 18.5: overall: aHR; 95% CI: 1.37; 1.12–1.69; women: 1.64; 1.25–2.13; men: 1.27; 0.95–1.69 BMI 25–29.9: aHR; 95% CI: 0.80; 0.69–0.92 BMI 30–34.9: aHR; 95% CI: 0.75; 0.60–0.93 BMI ≥35: aHR; 95% CI: 0.80; 0.64–1.02

8.		<23 in men >22.3 in women	Prospective study	14,833 aged ≥75 y, 90% non-smokers	Median: 5.9 y	All-cause mortality and cause-specific mortality	BMI <18.5 non-smoking men: HR; 95% CI: 2.03; 1.48–2.78 BMI <18.5 non-smoking women: HR; 95% CI: 1.35; 1.07–1.71 Circulatory mortality: BMI <18.5 non-smoking men: HR; 95% CI: 2.08; 1.14–3.83 BMI <18.5 non-smoking women: HR; 95% CI: 1.24; 0.90–1.70
9.	Winter et al. (2014)	<23 >33	Meta-analysis of 32 articles published from 1990 to 2013	197,940 Aged ≥65	Median: 12 y	All-cause mortality	Compared with BMI range of 23–23.9 BMI range 21–21.9: HR; 95% CI: 1.12; 1.10–1.13 BMI range 20–20.9: HR; 95% CI: 1.19; 1.17–1.22 BMI >33: HR; 95% CI: 1.08; 1.00–1.15 The highest all-cause mortality risk compared with reference range was found for BMI ranges <23 and ≥33

Abbreviations: *aHR* adjusted hazard ratio, *BMI* body mass index, *CI* confidence interval, *CT* computed tomography, *CVD* cardiovascular disease, *HR* hazard ratio, *HUNT* North-Trøndelag Health Study, *LOS* length of stay, *OR* odds ratio, *TJA* total joint arthroplasty, *WC* waist circumference, *WHR* waist-to-hip ratio

populations (Anaszewicz and Budzyński 2017; Tojek et al. 2019; Spychalska-Zwolińska et al. 2020; Wawrzeńczyk et al. 2019), although such prognostic power was found for other single (e.g., blood albumin concentration, total lymphocyte count, and total and LDL cholesterol), and composite indices of nutritional risk and status assessment (e.g., NRS-2002) (Budzyński et al. 2016).

Normal BMI range, overweight, and Class I obesity were associated with better patient prognosis than a diagnosis of malnutrition or underweight, which is represented by the middle of the J-shaped associations between BMI value and all-cause mortality mentioned above (Budzyński et al. 2016; Tojek et al. 2019). The U-shape association between BMI and mortality has also been previously reported in the Third National Health and Nutrition Examination Survey (NHANES III) population (Romero-Corral et al. 2008). For this reason, the ESC recommended a BMI of 20–25 kg/m^2 (for individuals aged below 60 years of age) and a WC for males of $<$94 cm and $<$80 cm for females as desirable ranges of nutritional biomarkers in the prevention of cardiovascular diseases (Visseren et al. 2021). Among populations of Asian ethnicity, BMI cut-offs related to lower all-cause mortality were generally lower than in other populations (Visseren et al. 2021). However, in older age, not only overweight, but even obesity, as defined by BMI, might protect against mortality (Sommer et al. 2020). Sommer et al. (2020) reported that being overweight (BMI 25–29.9 kg/m^2) was associated with reduced risk of all-cause mortality (HR; 95% CI: 0.94; 0.91–0.96), and Class I obesity (BMI 30–34.9 kg/m^2) was not related significantly with higher mortality (0.95; 0.88–1.01). Whereas, in a study by Streng et al. (2017), higher BMI was associated with lower mortality rates up to 180 days, but only in univariable analysis (HR: 0.53). Compared with normal weight and malnourished patients, some mildly obese patients with atrial fibrillation (HR: 0.41–1.42), CHF (HR: 0.47–1.69), and PAD (HR: 0.37–1.09) also showed a better prognosis, suggesting the existence of a BMI paradox (Anaszewicz and Budzyński 2017; Spychalska-Zwolińska et al. 2018; Wawrzeńczyk et al. 2019). Similarly, in critically ill COVID-19 patients with respiratory failure in the course of acute respiratory distress syndrome, BMI $\geq$30 kg/m^2 (HR; 95% CI: 0.32; 0.20–0.51) (Caccialanza et al. 2021) and even moderate obesity (BMI 30–39.9 kg/m^2) also had a degree of protection against in-hospital mortality compared with the other patients (Dana et al. 2021). These data are in some contrast to obesity (BMI $\geq$30 kg/m^2) being considered as a risk factor for death among COVID-19-positive patients. A more severe course of COVID-19 in obese patients is explained by impairments in the adaptive immune response, cardio-metabolic and thrombotic complications, and restrictive abnormalities of lung function due to visceral fat distribution, and higher prevalence of obesity-related co-morbidities.

Some authors suggest that the utility of the use of high BMI ($\geq$30 kg/m^2) as a biomarker of a patient's prognosis may be affected by adipose type distribution (not only android and gynoid, but also epicardial, perivascular, etc.), lean body mass expressed as FFMI (lean mass index) (Han et al. 2010), dysregulation in adipocytokine secretion by adipose tissue (Wawrzeńczyk et al. 2019), and the presence of the metabolic obesity consequences mentioned above; that is, the obesity paradox, MHO, MUHO, NWO, MONW, and sarcopenic obesity (Bragança et al.

2020; Visseren et al. 2021). Of those disturbances, sarcopenic obesity is associated with a worse patient prognosis, and the obesity paradox does not concern all health conditions. Johnson et al. (2021) found that among patients with ulcerative colitis, the prevalence of obesity increased two- to threefold over the 40-year study period. Compared with patients of normal weight, those with obesity had increased risk of hospitalization (HR; 95% CI: 1.72; 1.10–2.71) and, with each incremental increase in BMI of 1 kg/m^2, the risk of hospitalization increased by 5% (1.05; 1.01–1.08) and the risk of corticosteroid use increased by 2.6% (1.026; 1.00–1.05). Similarly, in a study by Ko et al. (2021), in multivariable analysis of 1225 primary invasive breast cancer patients and 35,991 healthy females, individuals with overweight or obesity had worse disease-free survival (HR; 95% CI: 1.98; 1.34–2.92) than underweight or normal weight individuals, and patients with high BMI and low total lymphocyte count had the shortest disease-free survival (2.48; 1.70–3.62). However, among 3661 East Asian patients undergoing cardiovascular surgery, the risks of all-cause and cardiac-cause mortality decreased with an increase in subcutaneous fat measured from cross-sectional CT images (adjusted HR; 95% CI: 0.997; 0.994–1.000 and 0.994; 0.989–0.999, respectively). In this study, paradoxically, no association was detected between total and visceral fat area and mortality (Bae et al. 2021).

Further Study

The state of the knowledge concerning the usefulness of BMI as a biomarker of patients' nutritional health demonstrated above shows a potential need to update age-specific BMI cut-offs for children, adolescents, adults, and the elderly, both in the general, healthy ambulatory population and for inpatients (Ng et al. 2017; Javed et al. 2020). There is also a need for further study of the determination of the cut-off for BMI as a biomarker of malnutrition. Further exploration is also needed to assess the utility of BMI as a biomarker of metabolic alterations associated with malnutrition, overweight, and obesity, as well as a biomarker of patients' prognosis with regard to hard and soft end-points during short- and long-term observation periods. It would also be useful to determine the relationships between BMI value and the findings of body imaging (e.g., ultrasonography, CT, and MRI) and types of body composition analysis (e.g., using different frequencies in BIA or DXA) and compare their predictive and prognostic utility. Moreover, a very important issue seems to be the clinical significance of BMI fluctuation in short- and long-term observation periods, particularly in the light of the increasing number of bariatric surgery and/or bariatric endoscopy procedures performed. Some doubts concerning the safety of these procedures are evoked by data showing that: (a) an increase in BMI is associated with lower mortality risk compared with a decrease in BMI (HR: 0.83–0.95) (Javed et al. 2020); (b) body weight fluctuations or weight loss are associated with increased risk of all-cause mortality (pooled HR: 1.41–1.45; 95% CI: 1.27–1.58) and CVD mortality (1.36–1.50; 1.22–1.70), increased CVD morbidity (1.49; 1.26–1.76), and risk of hypertension (1.35; 1.14–1.61) (Karahalios et al. 2017; Zou et al. 2019; Alharbi et al. 2021). In this context, what is also very

important is clarification of the existence and clinical importance of the obesity paradox, which seems to be adiposity-, distribution-, and age-specific (Javed et al. 2020).

Conclusions

BMI is a simple parameter of nutritional status assessment that has a number of limitations. Widely accepted BMI cut-offs show low sensitivity and high specificity for detecting BF%, which can lead to obese patients being overlooked and a consequent failure to provide them with health-promoting advice. Therefore, validation and generalization of new and simple methods of body composition analysis are required. The diagnostic and prognostic importance of both low and high BMI, especially with regard to nutritional, cardiovascular, and cancer-associated prognoses, both among out- and inpatients, is unclear, and, therefore, the use of BMI as a biomarker for such purposes should be carried out with care, taking under consideration its limitations.

Key Facts of BMI as a Biomarker in Patients' Nutritional Assessment

- BMI is a simple parameter of nutritional status assessment with a lot of limitations with regard to diagnosis of both malnutrition and obesity.
- Widely accepted BMI cut-offs show low sensitivity and high specificity for detecting percentage of body fat and nutritional abnormalities, which could lead to both obese and malnourished patients being overlooked and the opportunity of giving them health- promoting advice being missed. This limitation also indicates the need to interpret studies based on BMI with care.
- Low BMI is not useful as a single marker in the detection of malnutrition and/or decrease in lean mass related to chronic and acute diseases.
- BMI affects blood concentrations of cardiovascular risk biomarkers, which can lead to the misdiagnosis of some health conditions that are not related to nutritional status, such as an underdiagnosis of CHF due to a negative BMI correlation with NT-proBNP.
- BMI in the range of 20–25 kg/m^2 is related to the lowest all-cause mortality, and both lower and higher BMI values, as well as BMI fluctuations, are related to worse prognoses.

Mini-Dictionary of Terms

- Accuracy – The difference between test results and the true value.
- Biomarker – Any parameter (substance, structure, or process) that measures the body's homeostasis and predicts the occurrence of an outcome or disease.

- BMI – Calculated as a ratio of a patient's weight in kilograms and his or her height in meters squared.
- Confidence interval (95% CI) – The range of parameter values that contain 95% of the true population measurements.
- Hazard ratio – The risk outcome as a proportion in exposed and non-exposed populations.
- Negative predictive value (NPV) – The percentage of true negative results among all the tests performed.
- Positive predictive value (PPV) – The percentage of true positive results among all the tests performed; also known as precision.
- Sensitivity – The percentage of patients diagnosed with a condition made on the basis of the gold standard who achieved a positive test (e.g., BMI $\geq$30 kg/m^2).
- Specificity – The percentage of patients diagnosed as not having a condition on the basis of the gold standard who achieved a negative test (i.e., BMI $<$30 kg/m^2 when BMI $\geq$30 kg/m^2 was established as a positive test).

Summary Points

- BMI has numerous limitations as a biomarker of a patient's nutritional status assessment, a risk factor for diseases and health conditions, and a biomarker of all-cause mortality.
- BMI ranges defining malnutrition, overweight, and obesity need to be updated for the general population, for in- and outpatients, in relation to age ranges, and purpose of use; e.g., whether it is used as a risk factor for disease or as a prognostic factor for disease course or outcome among patients with different health conditions.
- BMI explain only small part variance of the other biomarkers for cardiovascular, metabolic, and inflammatory disorders.
- BMI is not useful as biomarker for monitoring of effectiveness both nutritional support and treatment among malnourished patients, and weight lowering therapy among obese individuals.
- The J-shaped relationship between BMI and risk of nutrition-related disease is shifted to the right for all-cause mortality; this is known as the obesity paradox.

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Three-Dimensional Ultrasound for Sensitive Assessment of the Effects of Nutritional Therapy on Carotid Atherosclerosis

30

Bernard Chiu, Yuan Zhao, and Xueli Chen

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Abstract

Dietary supplements have a much smaller effect on atherosclerosis compared to powerful medical therapies such as high-dose statins. Therefore, sensitive and cost-effective biomarkers are required to evaluate the efficacy of such supplements. We present two methods for assessing the effect of nutritional therapy (pomegranate juice/tablets) using three-dimensional ultrasound imaging. The first is a plaque texture biomarker representing the changes of 376 plaque textural features. The second measures the weighted average vessel-wall-plus-plaque thickness (VWT), computed by taking an average of the VWT-Change distribution weighted by a map highlighting anatomic locations likely to exhibit plaque changes. The two biomarkers were able to detect the difference between the treatment and placebo groups in a clinical trial evaluating the effect of pomegranate. These biomarkers reduce the number of subjects required to establish

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significance in a 1-year study by more than one order of magnitude compared to total plaque volume. The reduction in the cost required to evaluate new treatments will shorten the period that effective treatments are withheld from patients.

Keywords

Three-dimensional ultrasound (3DUS) · Carotid atherosclerosis · Pomegranate therapy · Plaque texture · Vessel-wall-plus-plaque thickness (VWT)

Abbreviations

3DUS	Three-dimensional ultrasound
ALS	Arc-length scaling
CCA	Common carotid artery
DL	Description length
GLCM	Gray-level co-occurrence matrix
GLD	Gray-level distribution
ICA	Internal carotid artery
IMT	Intima-media thickness
LADA	Locality alignment discriminant analysis
LBP	Local binary pattern
LDA	Linear discriminant analysis
LDL	Low-density lipoproteins
LIB	Lumen-intima boundary
LPP	Locality-preserving projections
MAB	Media-adventitia boundary
MDL	Minimum description length
MI	Mutual information
NGTDM	Neighborhood gray tone different matrix
PCA	Principal component analysis
TPV	Total plaque volume
VWT	Vessel-wall-plus-plaque thickness
$\overline{\Delta VWT}_{\text{Weighted}}$	Weighted vessel-wall-plus-plaque thickness average
VWV	Vessel wall volume

Introduction

Stroke is among the leading causes of death worldwide, with 5.5 million stroke-related deaths in 2016 (Johnson et al. 2019). Carotid atherosclerosis is a major source of atherosclerotic emboli that would block cerebral arteries and thus lead to ischemic strokes. It was estimated that for high-risk patients, 75–80% of strokes could be prevented by lifestyle/dietary changes and medical therapies (Spence 2007). In parallel to the development of novel therapies, the development of sensitive and cost-effectiveness measurement tools is required for sensitive serial monitoring of carotid atherosclerosis.

Carotid intima-media thickness (IMT) measured from the longitudinal view of two-dimensional ultrasound images has been shown to correlate with an increased risk of vascular events (Bots and Grobbee 2002). However, IMT is genetically (Pollex and Hegele 2006), biologically (Spence 2015) and pathologically (Finn et al. 2010) distinct from atherosclerosis. Furthermore, as the annual change rate of IMT is small (~0.015 mm), the difference between treatment and placebo groups could not be demonstrated without a large number of patients followed over an extended period. Additionally, IMT is measured from two-dimensional ultrasound images, the acquisition of which requires an operator to select a plane to the image. This requirement limits the reproducibility of 2DUS imaging, making it suboptimal for serial monitoring of plaque changes in longitudinal analysis.

Measurements from three-dimensional ultrasound (3DUS) are more sensitive to changes as plaques grow longitudinally, circumferentially, and in thickness. Total plaque volume (TPV) (Ainsworth et al. 2005) and vessel wall volume (VWV) (Krasinski et al. 2009b) were shown to be able to detect the difference between patients randomized to intensive atorvastatin versus placebo in a 3-month clinical trial. However, few therapies are this powerful, and for ethical reasons, most therapies would be assessed on the background of cardiovascular therapies. There is a crucial need for biomarkers very sensitive to treatment effect since the background medications may obscure the small benefits conferred by dietary supplements, such as pomegranate.

Pomegranate is a dietary supplement shown to inhibit low-density lipoprotein (LDL) oxidation and attenuate atherosclerosis development in animal studies (Aviram 2000; Fuhrman and Aviram 2001). The presence of polyphenols ellagitannins and ellagic acid, anthocyanins and flavonoids, equips pomegranate with vasculoprotective effects. Anti-inflammatory Urolithin A (Fu et al. 2019) and insulin secretion-promoted Urolithin C (Bayle et al. 2019) were included in metabolites of these polyphenols. Although the antiatherosclerotic effect of pomegranate has been shown in animal studies, it was found in a recent 18-month randomized placebo-controlled trial involving 289 subjects that the IMT measured from subjects treated by pomegranate was not different from the control group ($p = 0.65$) (Davidson et al. 2009). However, the finding may result from the relative insensitivity of IMT to treatment effect. In this chapter, two sensitive biomarkers are introduced that are able to show the effect of pomegranate juice or tablet.

The presence of vulnerable plaques increases the risk of thrombogenic events leading to ischemic stroke. Plaque stability is determined by composition, which can be characterized through the plaque texture measurements in 3DUS. Awad et al. (2010) classified patients who received high-dose atorvastatin and placebo based on 270 plaque textural features extracted from each slice of 3DUS images and found that the classification accuracy associated with plaque textural features is higher than TPV. Van Engelen et al. (2014) predicted cardiovascular events of patients based on 376 textural features and concluded that plaque textural change is more accurate than TPV-Change (ΔTPV) in predicting cardiovascular events. Chen et al. (2020) developed a sensitive plaque texture-based biomarker that quantifies the amount of plaque texture changes in a year and demonstrated that the sample size required to show the

effect of pomegranate was 20 times smaller than ΔTPV. This biomarker is discussed in detail in section "Plaque Texture Analysis."

Carotid atherosclerosis is a focal disease, and we have demonstrated that assessment by local vessel-wall-plus-plaque thickness change (ΔVWT) is more sensitive than VWV-Change (ΔVWV) in detecting treatment effect (Cheng et al. 2017). To allow direct and pointwise VWT quantification across carotids with variable geometry, we developed a technique to map the VWT distribution for each patient to a carotid atlas with standardized geometry (Chiu et al. 2013; Chen and Chiu 2016). An important criterion of a sensitive biomarker is the ability to focus on regions with substantial change. We developed a weighting metric that highlights the anatomic locations likely to exhibit change (Cheng et al. 2017). This metric was used to compute the weighted average of ΔVWT ($\overline{\Delta VWT}_{\text{Weighted}}$), which were shown to reduce the sample size required to detect the effect of B-Vitamin supplement by 71%, as compared to ΔVWV. We also showed that $\overline{\Delta VWT}_{\text{Weighted}}$ was able to detect the effect of pomegranate in a placebo-controlled clinical trial (Zhao et al. 2021). This study is detailed in section "Vessel-Wall-Plus-Plaque Thickness (VWT) Analysis" of this chapter.

Quantitative Assessment of Treatment Effect Based on 3D Carotid US Images

Plaque Texture Analysis

Plaque Segmentation and Texture Extraction

Chen et al. (2020) investigated a cohort recruited in a placebo-controlled study with 96 subjects taking pomegranate extract in a tablet/juice once daily and 75 subjects taking a placebo-matching tablet/juice once daily. Participants were scanned at baseline before the study and 376 $\pm$ 23 days (range: 283–579 days) later.

Carotid plaques in 3DUS images were segmented following the workflow described in Egger et al. (2007) by a trained observer. The intensities of the images were linearly rescaled so that the 10th and 90th percentile was set at a gray-level of 10 and 150, respectively. A set of 376 textural features from 9 different texture extraction techniques was generated for each plaque in the left and right arteries as described in van Engelen et al. (2014). The nine texture extraction techniques included the following: (1) gray-level distribution (GLD), (2) gray-level co-occurrence matrix (GLCM), (3) gray-level run-length matrix, (4) gray-level difference statistics, (5) neighborhood gray tone different matrix (NGTDM), (6) laws texture, (7) local binary pattern (LBP), (8) Gaussian filter bank, and (9) structure tensor. For each feature, a subject average was computed by weighting the value computed for each plaque with its total plaque volume (TPV), and thereby one measurement per subject was obtained at each of the baseline and follow-up sessions. Feature changes for all patients were normalized to their annual rate. The annual feature changes were further normalized based on the min-max scaling technique.

Dimensionality Reduction and Biomarker Generation

Since the number of features (376) was greater than the number of subjects (171) in this study, principal component analysis (PCA) was first used to reduce the dimension of the feature set. Horn's parallel analysis (Horn 1965) was applied to determine the number of principal components in the PCA model. Locality preserving projections (LPP) (He and Niyogi 2004) was further applied to weight elements in the reduced feature vectors produced by PCA. LPP produces an optimal linear approximation that captures essential patterns exhibited locally in the feature space. It preserves local structures relating to the proximity of data points in the low-dimensional manifold by imposing a heavy penalty if a pair of neighboring points $\mathbf{x_i}$ and $\mathbf{x_j}$ having a high similarity a_{ij} are mapped far apart. a_{ij} are defined as cosine similarity:

$$a_{ij} = \begin{cases} \cos\left(\mathbf{x_i}, \mathbf{x_j}\right) \textit{ if } \mathbf{x_i}, \mathbf{x_j} \text{ are in the same group} \\ 0 \text{ elsewhere} \end{cases} \tag{1}$$

The optimization problem is formulated as:

$$\min_{\mathbf{w}} \frac{1}{2} \sum_{\mathrm{i,j}} a_{ij} \left(\mathbf{x_i}^{\mathrm{T}} \mathbf{w} - \mathbf{x_j}^{\mathrm{T}} \mathbf{w} \right)^2. \tag{2}$$

It can also be written in a matrix form:

$$\min_{\left\{\mathbf{w}:\mathbf{w}^{\mathrm{T}}\mathbf{X}\mathbf{D}\mathbf{X}^{\mathrm{T}}\mathbf{w}=\mathbf{1}\right\}} \mathbf{w}^{\mathrm{T}}\mathbf{X}\mathbf{L}\mathbf{X}^{\mathrm{T}}\mathbf{w}, \tag{3}$$

where $\mathbf{X} = [\mathbf{x_1}, \mathbf{x_2}, \cdots, \mathbf{x_{171}}]$. $\mathbf{L}$ is the Laplacian matrix, defined as $\mathbf{D} - \mathbf{A}$, where $\mathbf{A} = \{a_{ij}\}$ and $\mathbf{D}$ is a diagonal matrix with $d_{ij} = \sum_j a_{ij}$ and is known as the degree matrix. The solution can be obtained by performing eigenvalue decomposition of the matrix $(\mathbf{X}\mathbf{D}\mathbf{X}^{\mathbf{T}})^{-1}\mathbf{X}\mathbf{L}\mathbf{X}^{\mathbf{T}}$.

LPP is a supervised method as the determination of the similarity coefficient a_{ij} (Eq. (1)) requires the group label information. Leave-one-out cross-validation was applied in this study. In each of the 171 leave-one-out trials, a biomarker was generated for a single subject while the features of the remaining 170 subjects were used to train the biomarker. In this way, a scalar biomarker was generated for each of the 171 subjects.

The sensitivity of LPP in detecting treatment effect was compared with two supervised dimensionality reduction techniques, known as the linear discriminant analysis (LDA) and locality alignment discriminant analysis (LADA), and total plaque volume change (ΔTPV) measured from 3DUS. TPV has been shown to be reproducible (Egger et al. 2007) and capable of predicting vascular events (Wannarong et al. 2013). Two-sample t-tests were conducted to evaluate the discriminative power of the LDA, LADA, LPP biomarkers, and ΔTPV. Table 1 shows the results associated with ΔTPV, the LDA, LADA, and LPP biomarkers. These biomarkers were also compared with the standard deviation of homogeneity derived

Table 1 The means and standard deviations (in parentheses) of ΔTPV, the most discriminative feature among the 376 features (GLCM Coronal std. homo), the LDA, LADA, and LPP biomarkers computed for the placebo and pomegranate groups and the P-values associated with two-sample t-tests

Measurements	Placebo ($n = 75$)	Pomegranate ($n = 96$)	P-value
ΔTPV (mm^3)	28 (77)	17 (77)	0.34
GLCM Coronal std. homo	0.64 (0.16)	0.73 (0.15)	4.8×10^{-4}
LDA	0.31 (1.39)	−0.19 (1.02)	7×10^{-3}
LADA	0.14 (0.50)	−0.13 (0.40)	1.8×10^{-4}
LPP	0.23 (0.42)	−0.07 (0.46)	1.5×10^{-5}

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Table 2 Sample sizes per group required for various effect sizes in a 1-year study. The sample sizes below give a 90% statistical power and a significant level of 0.05 (two-tailed). The effect sizes are expressed as the percentage of the current placebo-controlled study

Effect size	ΔTPV	GLCM: Coronal, std. homo	LDA	LADA	LPP
100%	971	70	124	58	45
75%	1726	125	220	104	80
50%	3883	281	495	233	180

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from GLCM in the coronal plane (GLCM Coronal std. homo), which is the most discriminative plaque textural feature among the 376 features. Sample size estimation was computed to evaluate the cost-effectiveness of the biomarkers. Table 2 shows the sample size required for the five biomarkers to detect various effect sizes (i.e., 50%, 75%, and 100% of that exhibited in the current placebo-controlled trial) in a 1-year study. The proposed requires 20 times fewer subjects than ΔTPV to detect the effect of pomegranate.

Physical Understanding of the Biomarker

To understand the biomedical meaning behind the proposed biomarker, a metric was introduced in Chen et al. (2020) to evaluate how much each of the 376 features contributed to the proposed biomarker. The importance of the j^{th} eigenvector obtained in PCA (i.e., the 376-dimensional $\mathbf{\Phi_j}$) is quantified by the j^{th} component of $\mathbf{w}^*$ obtained using a dimensionality reduction method (e.g., LDA, LADA, or LPP). Therefore, the strengths of each textural feature d (denoted by $S(d)$) can be quantified by the weighted sum of N_x eigenvectors, using the following equation:

$$S(d) = \sum_{j=1}^{N_x} w_j \Phi_j(\mathrm{d}) \; for \; d = 1, 2, \ldots, 376, \tag{4}$$

where w_j is the j^{th} component of $\mathbf{w}^*$, which is the weighting vector generated by a dimensionality reduction method. The number of eigenvectors involved, N_x, was obtained from Horn's parallel analysis. The average absolute value of $S(d)$s from the

Table 3 Feature ranking for the LPP model. H4N3: the normalized number of voxels in the plaque having 4 transitional areas in the 26-voxel neighborhood; HxN5: the normalized number of voxels in the plaque having x transitional areas in the 98-voxel neighborhood, where x = 6, 7, 8

Texture change	Weights	Rank order
LBP		
H4N3	0.17	1
H7N5	0.16	2
H8N5	0.12	4
H6N5	0.10	8
GLD		
Histogram 2	0.15	3
Histogram 3	0.11	6
Mode	0.11	7
NGTDM		
Contrast	0.12	5

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171 leave-one-out trials serves as a metric on the importance of textural feature *d* in a biomarker generated by a dimensionality reduction method.

Table 3 shows the top eight features with the highest absolute average weights from the 171 leave-one-out trials of LPP. In agreement with our findings, many of these top features were found to be strong features in risk stratification and treatment effect evaluation in previous studies. Christodoulou et al. (2003) found that the NGTDM technique performed best in classifying symptomatic and asymptomatic subjects. van Engelen et al. (2014) found that NGTDM features were strongest in discriminating symptomatic and asymptomatic patients. LBP features were among the top features for discriminating symptomatic and asymptomatic patients in a study based on 2D longitudinal ultrasound images (Acharya et al. 2012). Histogram features and first-order statistics from GLD were able to predict plaque composition in a histological study (Rakebrandt et al. 2000). Many of these top features are associated with homogeneity and echogenicity. Heterogeneous and echolucent plaques consisted of intraplaque hemorrhage (Bluth et al. 1986) and calcification (Group 1995), contributing to the destabilization of plaques (Fisher et al. 2005), and thereby carry a higher neurological risk.

Figure 1 shows example plaques from a placebo subject and a pomegranate subject, with the LPP scores as 0.33 and −0.16, respectively. Three contiguous axial images of each plaque were shown. The GLD mode decreased for the placebo subject and increased for the pomegranate subject. This indicates that the plaque of the placebo subject had become more echolucent, whereas that of the pomegranate subject had become more echogenic, which can be visually observed in the regions pointed by the arrowheads. Similar observations can be found according to the changes of the normalized histogram in the low-intensity spectrum (Histogram 2 and 3). LBP detected textural patterns characterized by microstructures such as edges, lines, spots, and flat areas, by quantifying the number of transitions from a higher to a lower intensity with respect to the center voxel (Ojala et al. 2002). The four LBP features decreased more in the placebo plaque and only changed little in the pomegranate plaque as shown in Fig. 1, indicating a more stable textural

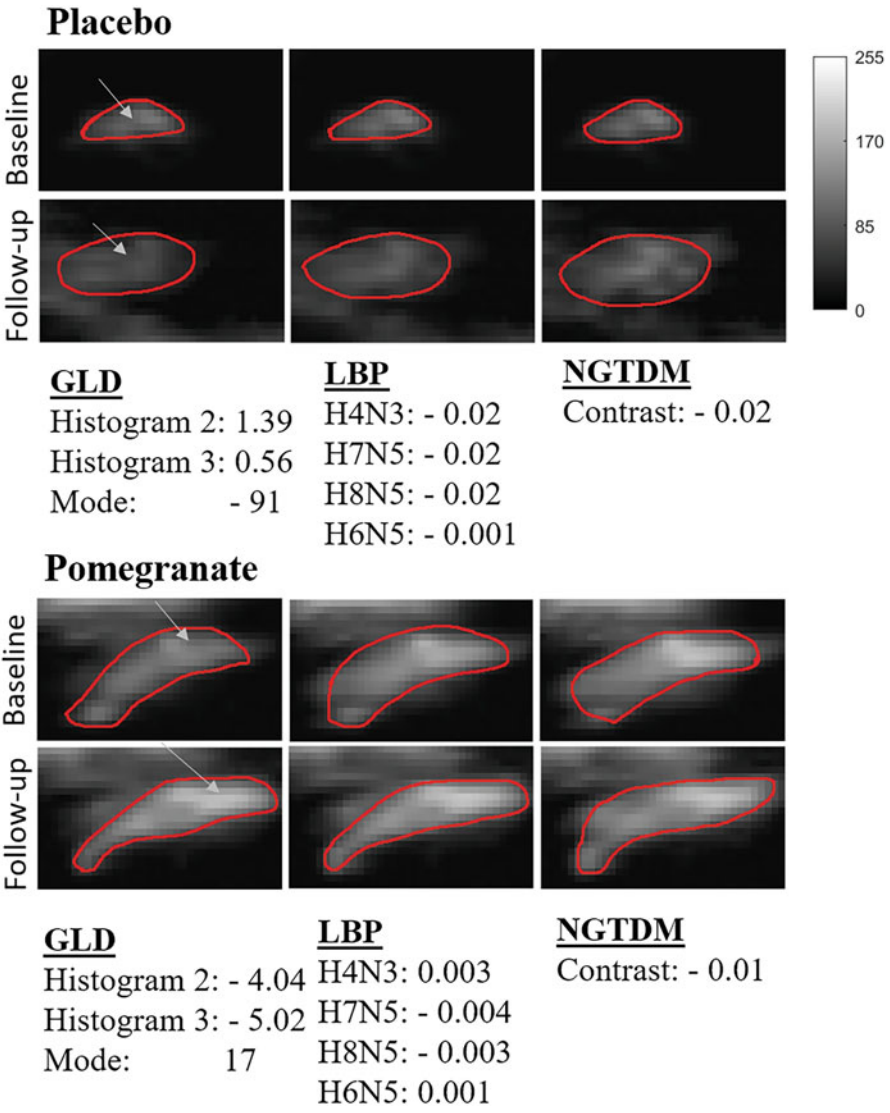

Fig. 1 Example plaques for a placebo and pomegranate subject with strong textural features listed. The first and third rows show the plaques in baseline, and the second and fourth rows show the plaques in follow-up. The difference in echogeneity between baseline and follow-up was shown for the two example plaques using arrowheads. The LPP scores for the placebo and the pomegranate subjects are 0.33 and −0.16, respectively. H4N3: the normalized number of voxels in the plaque having four transitional areas in the 26-voxel neighborhood; HxN5: the normalized number of voxels in the plaque having x transitional areas in the 98-voxel neighborhood, where x = 6, 7, 8. (From Chen et al. (2020) with permission)

pattern for the pomegranate plaque. The NGTDM contrast introduced in Amadasun and King (1989) is associated with the dynamic range of grayscale, the amount of local intensity variations, and the total number of voxels of the plaque. High contrast is attained with a large dynamic range and a large local intensity variation in a small volume. In the example plaques shown in Fig. 1, the feature decreased more in the placebo plaque than in the pomegranate plaque, although the difference was small. However, the difference in the longitudinal change of the image contrast exhibited in the two example plaques seems to be more substantial, as shown in Fig. 1. We found that the involvement of plaque volume in the calculation of NGTDM contrast has confounded the textural difference exhibited in the two plaques, with plaque volume increased by 53 mm^3 for the placebo plaque and only 4 mm^3 for the pomegranate plaque. This observation illustrates that although NGTDM contrast contributed to the discrimination power of the LPP biomarker, a single feature would not be able to detect the textural differences exhibited in all individual patients. It was exactly for this reason that biomarkers were developed in this study to integrate the discrimination power of a large number of textural features.

Vessel-Wall-Plus-Plaque Thickness (VWT) Analysis

The cohort involved in the randomized placebo-controlled study of the effects of pomegranate juice/tablets were analyzed using a VWT-based biomarker in Zhao et al. (2021). Figure 2 shows the workflow for computing $\overline{\Delta VWT}_{\text{Weighted}}$ for each subject. Details of each step are described in the following subsections.

Generation of 2D VWT Map

VWT is the distance between the lumen-intima boundary (LIB) and the media-adventitia boundary (MAB). To calculate VWT, the carotid LIB and MAB are required to be segmented from each 2D axial image resliced from the 3DUS volume. The trained observer delineated LIB and MAB of the common and internal carotid arteries (CCA and ICA, respectively) on each 2D resliced image, as shown in Fig. 2a. The two boundary contours (i.e., LIB and MAB) in each 2D resliced image were matched on a point-by-point basis to compute the point-wise VWT measurements, which were superimposed on the MAB surface, resulting in the 3D VWT map shown in Fig. 2b.

The flow chart for constructing the 2D VWT map is shown in Fig. 3. The initial 2D VWT map was generated by the arc-length-scaling (ALS) approach (Chiu et al. 2013), which provided an initial mapping from $\mathbb{R}^3$ to $\mathbb{R}^2$ (Fig. 2c). Then, correspondence of the VWT maps for the same subject and across subjects was optimized by minimization of description length (DL) (Chen and Chiu 2016) to generate the optimized 2D VWT map (Fig. 2d).

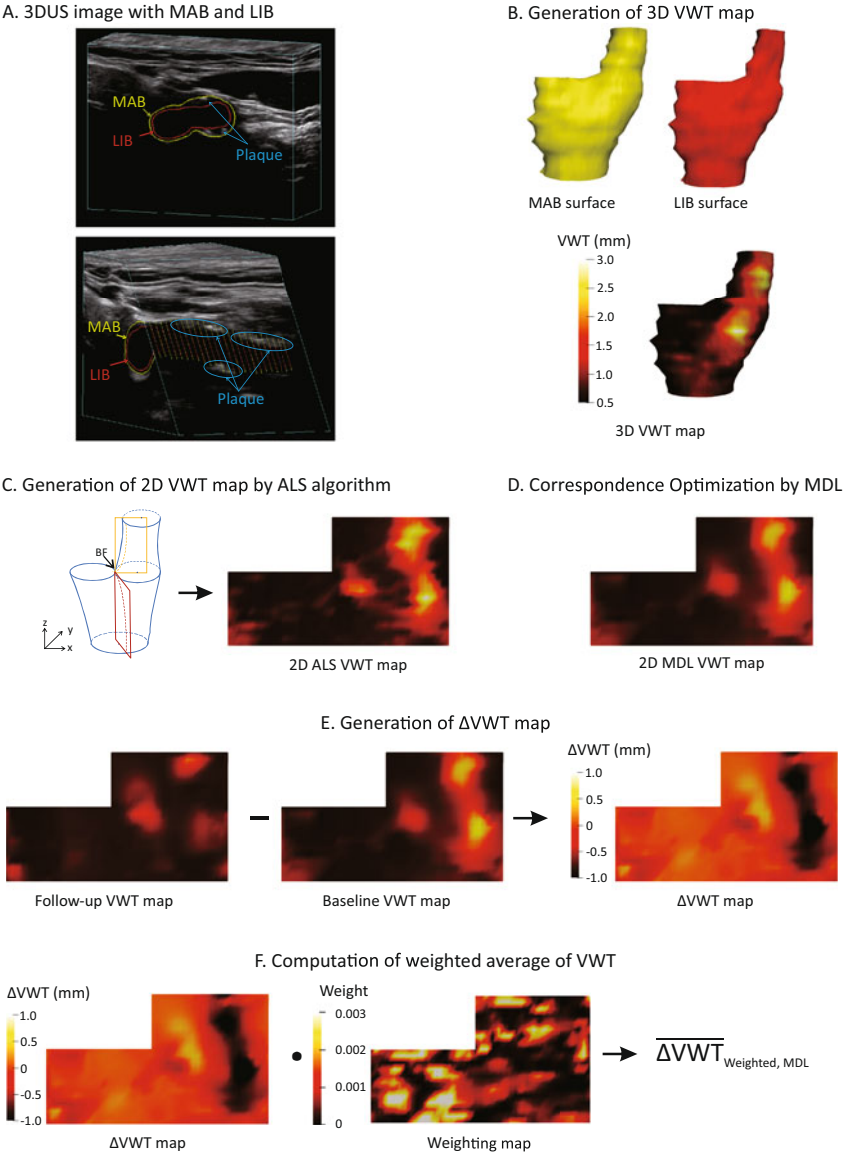

Fig. 2 The workflow for measuring the weighted average of carotid vessel-wall-plus-plaque thickness change. (**a**–**d**) show the four steps required to generate the 2D VWT map for each artery. The VWT-Change map was generated by taking the difference between the baseline and follow-up maps, as shown in (**e**). Pointwise VWT-Change measurements were weighted by a weighting map to produce the weighted average of VWT-Change as shown in (**f**). (From Zhao, Spence, and Chiu (2021) with permission)

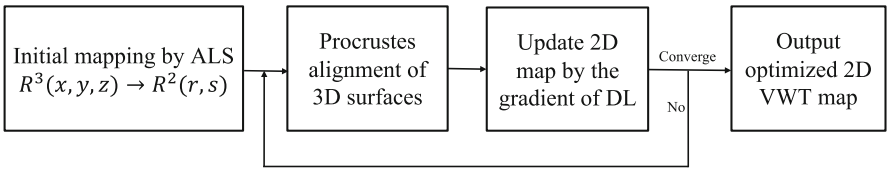

Fig. 3 The flowchart to generate the optimized 2D VWT map

The first step in mapping a 3D surface to a 2D space using the ALS approach involves aligning the 3D VWT map with the standard 3D coordinate system, in which the origin is located at the carotid bifurcation. The longitudinal direction of the carotid artery is aligned with the z-axis, and the positive z-axis points toward the downstream direction of flow. The vector pointing from the origin to the centroid of the ICA contour on the same plane was aligned with the x-axis. The y-axis was defined by taking the direction of the cross-product of the z-axis and x-axis (i.e., $\vec{z} \times \vec{x}$). Then, the CCA and ICA in the aligned 3D VWT map were cut by two planes (Fig. 2c), and each unfolded to an L-shaped domain. Finally, each 2D map was sampled in a 0.5 mm interval in both vertical and horizontal directions to obtain the initial 2D VWT map, as shown in Fig. 2c.

The 2D VWT obtained by the ALS approach did not consider the quality of the correspondence. The potential misalignment of points across patients and in the baseline and follow-up maps for the same patient would introduce inaccuracy in the calculation of pointwise ΔVWT and mean ΔVWT measurements. To address this issue, we developed an iterative algorithm to optimize the anatomic correspondence, which was detailed elsewhere (Chen and Chiu 2016; Fenster et al. 2018) and briefly summarized here. The points on the initial 2D VWT map provided a set of N landmarks, which have a one-to-one mapping to the corresponding 3D map. The iterative minimum description length (MDL) algorithm consists of the following two steps and has a goal to minimize the description length of the statistical shape model generated according to the established correspondence. First, all 3D VWT maps were registered according to this set of correspondence points using the Procrustes algorithm. Second, the gradient of DL was computed at landmarks in the 2D maps; landmarks were moved in the gradient descent direction, resulting in a set of updated correspondence points. Procrustes' registration was performed based on the updated correspondence followed by the next iteration of DL minimization.

Figure 4 shows the 3D and 2D VWT maps obtained at baseline and follow-up scan sessions from an example subject who received pomegranate therapy. The second and third rows provide a visual comparison between the VWT map generated by the ALS and MDL approaches. The ΔVWT maps were normalized to their annual rate and displayed in the fourth row. There are three plaques in the carotid artery of this subject, which are marked with blue circles. VWT increase can be observed adjacent to regions with large plaque regression, as shown in Fig. 4g, which is also observed in a previous

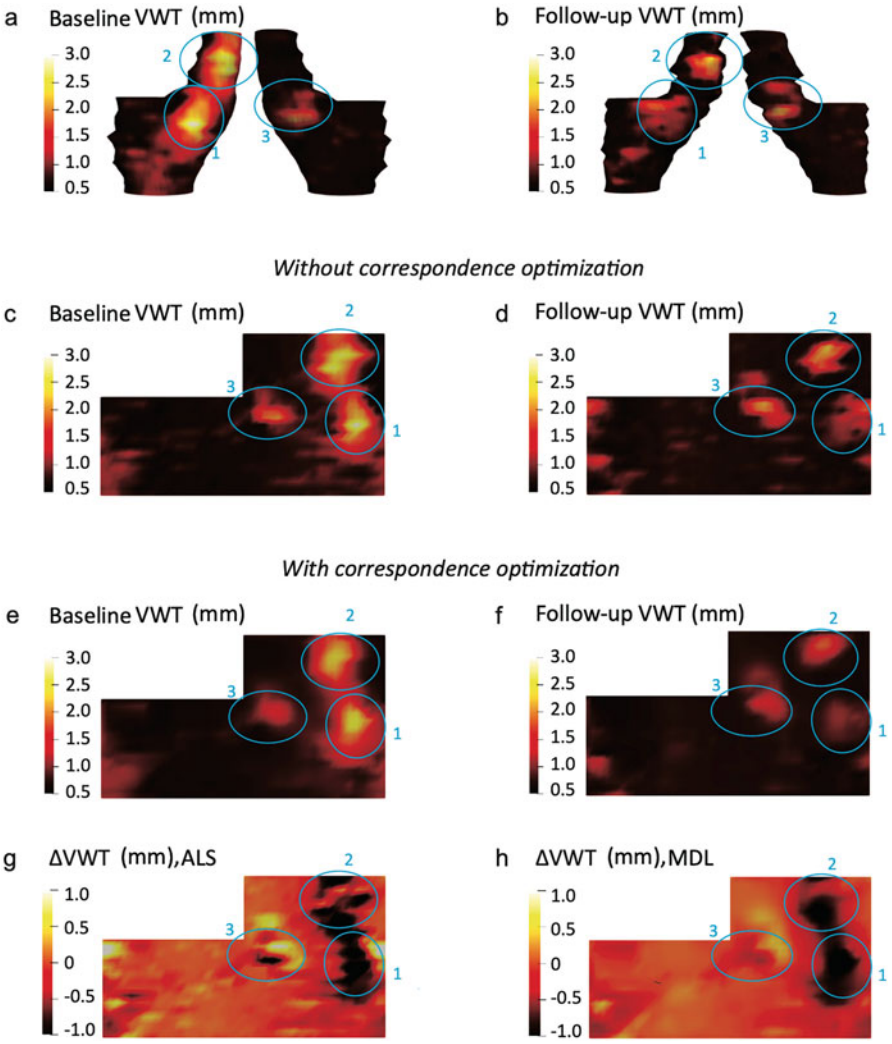

Fig. 4 The comparison between VWT maps generated with and without correspondence optimization. The 3D (**a**, **b**) and 2D VWT maps (**c**–**f**) of a subject in the pomegranate group. The blue circles highlight the three plaques in this artery. The right and left columns show maps generated at baseline and follow-up for the example subject. The second and the third rows show VWT maps generated without and with correspondence optimization, respectively. (**g**, **h**) show the annual VWT-Change maps generated without and with correspondence optimization, respectively. (From Zhao, Spence, and Chiu (2021) with permission)

study (Egger et al. 2008), but not in Fig. 4h. The difference between Figs. 4g and h suggests that the VWT increase shown in Fig. 4g may have been caused by the misalignment of the corresponding points in the baseline and follow-up VWT maps generated without correspondence optimization by the MDL approach.

Quantitative Assessment Based on $\overline{\Delta VWT}_{\text{Weighted}}$

Although quantitative measurement of pointwise ΔVWT obtained by taking the difference between the baseline and follow-up VWT maps allows spatial monitoring of vessel wall and plaque changes in individuals, the large number of pointwise ΔVWT measurements poses a challenge for clinical interpretation. For this reason, we proposed a biomarker based on the mutual-information-weighted average of ΔVWT, denoted by $\overline{\Delta VWT}_{\text{Weighted}}$, as shown in Fig. 2f. The weight map highlights the regions where plaques progression or regression is likely to exhibit. For subject j, the annual ΔVWT at position p_i on the 2D atlas is denoted by $f_j(p_i)$, and the treatment group to which the subject belongs is represented as C_j ($C_j = 0$ and $C_j = 1$ indicate placebo group and treatment group, respectively). $F(p_i)$ and C are the variables in a cohort with a realization $f_j(p_i)$ and C_j for each subject j, respectively. The weight $W(p_i)$ was developed based on mutual information (MI) in a cohort, quantifying the contribution of ΔVWT at each position p_i on the 2D template to the identification of the subject group C. The MI between $F(p_i)$ and C, denoted by $I(F(p_i); C)$, is high if the knowledge of $F(p_i)$ reduces much uncertainty about C (i.e., which treatment group a subject belongs to). Detailed description on how $I(F(p_i); C)$ was computed is provided in Cheng et al. (2017). The weighted average of subject j, denoted by $\overline{\Delta VWT}_{\text{Weighted},j}$, was computed by weighting the ΔVWT at point p_i by the weight $W(p_i) = I(F(p_i); C)$, expressed as follows:

$$\overline{\Delta VWT}_{\text{weighted},j} = \sum_{i=1}^{P} W(p_i) f_j(p_i), \tag{5}$$

where P is the total number of points on the 2D template.

The sensitivity of the biomarker was evaluated in a registered 1-year double-blind placebo-controlled clinical trial (ISRCTN30768139) (Zhao et al. 2021). Among 120 subjects involved in a placebo-controlled evaluation of the effect of pomegranate therapy on carotid atherosclerosis, 66 subjects received pomegranate tablets or juice as the pomegranate group, and the remaining 54 subjects received identical placebo tablets or juice. The subjects on cardiovascular medications are allowed to take background medication for ethical reasons and maintain the same regimen for the duration of the study. There were no significant differences between the pomegranate group and the placebo group in terms of baseline characteristics and background treatment medication. All participants had a 3D ultrasound scan of the carotid artery by the 3DUS imaging system described in Fenster, Downey, and Cardinal (2001). The weighting map used to calculate the biomarker was determined by an independent cohort participating in a 3-month randomized trial designed to study the effects of high-dose atorvastatin (Ainsworth et al. 2005). The cohort consisted of 34 subjects, of which 20 subjects received 80 mg atorvastatin tablet daily and 14 received identical placebo tablets. The P-values obtained in the two-sample t-tests were used as metrics to evaluate the ability of different biomarkers to discriminate different treatment groups. The means, standard deviations, and P-values of the biomarkers were listed in Table 4. For each biomarker, the

Table 4 The means, standard deviations, and P-values of the three biomarkers computed for the placebo and the pomegranate groups

Biomarkers	Pomegranate ($n = 66$)	Placebo ($n = 54$)	P-value
ΔTPV	15.84 (87.41)	25.79 (79.64)	0.53
$\overline{\Delta VWT}_{\text{weight},AL}$	0.010 (0.129)	0.062 (0.104)	0.017
$\overline{\Delta VWT}_{\text{weight},MDL}$	−0.006 (0.135)	0.057 (0.116)	0.008

From Zhao et al. (2021) with permission

Table 5 Sample size required to show the effects of pomegranate therapy for various effect size. The effect sizes are expressed as a percentage of those obtained in the current placebo-controlled study

Effect size (%)	ΔTPV	$\overline{\Delta VWT}_{\text{weight},AL}$	$\overline{\Delta VWT}_{\text{weight},MDL}$
100	1483	104	83
75	2636	185	148
50	5931	416	333
	Note: α = 0.05 (two-tailed), β = 0.1 (90% statistical power)		

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sample size required to detect a specific effect size δ was computed using the following equation:

$$n = \frac{\left(z_{\alpha/2} + z_{\beta}\right)^2 \left(\sigma_0^2 + \sigma_1^2\right)}{\delta^2} \tag{6}$$

where Z is a normally distributed random variable with zero mean and unity variance with $p(Z > z_{\beta}) = \beta$. σ_0 and σ_1 are the standard deviations of the biomarkers associated with the placebo and pomegranate groups, respectively. In this study, the sample size was computed at 90% statistical power (i.e., $\beta = 0.1$) and a significant level of $\alpha = 0.05$.

The sample size required for ΔTPV, $\overline{\Delta VWT}_{\text{Weighted},ALS}$, and $\overline{\Delta VWT}_{\text{Weighted},MDL}$ for different effect sizes were tabulated in Table 5. For a specific effect size, the sample size for $\overline{\Delta VWT}_{\text{Weighted},MDL}$ was 14 times lower than that for ΔTPV and was 20% lower than that for $\overline{\Delta VWT}_{\text{Weighted},ALS}$.

Discussion and Future Perspectives

Sensitive biomarkers for carotid atherosclerosis quantification are useful for monitoring high-risk patients and in proof-of-principle treatment effect evaluation. Dietary interventions are increasingly used as adjuvant treatments for cardiovascular diseases. The development of the biomarkers to detect the effect of such treatment is challenging because the beneficial effects of dietary treatments are expected to be weaker than intensive drug treatments, and most of these treatments are evaluated in the background of statin therapy, which makes the effect more difficult to detect.

In this chapter, two biomarkers were introduced that have sufficient sensitivity to detect the treatment effect of pomegranate on carotid atherosclerosis. In section "Plaque Texture Analysis," we developed a metric to combine 376 plaque texture measurements and showed that the resulting plaque texture-based biomarker can establish a significant difference between subjects receiving pomegranate and placebo. We showed that $\overline{\Delta VWT}_{\text{Weighted}}$ can also establish the effect of pomegranate in the same placebo-controlled study. Optimization of geometric correspondence between different VWT maps, generated either for different subjects or at different time points for the same subject, improved the sensitivity of the $\overline{\Delta VWT}_{\text{Weighted}}$ metric and reduced the sample size required to establish treatment efficacy by 20%.

The plaque texture-based biomarker required a sample size of 45 subjects to establish efficacy with a significance level of 0.05 and 90% statistical power (Chen et al. 2020), whereas 83 subjects were required to establish significance using $\overline{\Delta VWT}_{\text{Weighted},MDL}$. Therefore, biomarkers based on texture features can detect treatment effects more sensitively than biomarkers based on ΔVWT. However, plaque texture feature extraction requires plaque segmentation. Manual segmentation of plaques from 3D ultrasound images requires long training and implementation times. Spence and Parraga (2016) observed that one-third of observers are not able to segment plaques reliably. No automatic segmentation algorithm has been thoroughly validated for plaque segmentation from 3D ultrasound images, although semiautomatic plaque segmentation algorithms evaluated in small sample sets have been developed by us and others (Cheng et al. 2013, 2018). Therefore, the requirement for plaque segmentation precludes a wider application of texture-based biomarkers. In contrast, MAB and LIB can be segmented automatically from 3D ultrasound images using deep learning methods (Tan et al. 2020; Zhou et al. 2019). As $\overline{\Delta VWT}_{\text{Weighted}}$ only requires MAB and LIB, it can be readily integrated into the clinical workflow.

Despite practical limitations associated with the plaque texture-based biomarker, the success in our development of the texture-based biomarker suggests that 3DUS textural features are sensitive to treatment effect. Vessel wall and plaque textural features can be used instead, the extraction of which only requires the MAB and LIB. These features can be combined with VWT maps to produce a new biomarker with higher sensitivity than $\overline{\Delta VWT}_{\text{Weighted}}$. The weighting mechanism can be used to highlight both ΔVWT and textural features.

Deep learning algorithms have been used to analyze carotid artery ultrasound images. The applications of deep learning in the detection and analysis of carotid atherosclerosis include automatic extraction of biomarkers such as IMT (Shin et al. 2016), total plaque area (Zhou et al. 2021), VWV (Zhou et al. 2019), and automatic plaque component analysis for identifying vulnerable plaques (Lekadir et al. 2016). These applications used deep learning algorithms to learn abstract high-level representations of input data that are optimal for various learning tasks. Deep learning approaches can also be used to generate VWT-based biomarkers directly from the VWT maps. The vessel wall and plaque texture features can also be mapped to the carotid atlas and integrated into deep learning networks to produce a new sensitive biomarker.

Applications to Other Anti-atherosclerotic Treatments/Interventions and Image Modalities

In this chapter, we reviewed two 3DUS biomarkers for sensitive detection of treatment effect. Although the biomarkers were evaluated in a placebo-controlled clinical trial of the effect of pomegranate, they can be used to evaluate the effects of other dietary supplements and various dietary regiments (Shai et al. 2010) on the progression/regression of carotid atherosclerosis. The biomarkers can also be used to stratify stroke risk. For example, the biomarkers can be evaluated for their ability to predict cardiovascular events in a cohort described in van Engelen et al. (2014) consisting of asymptomatic subjects and subjects who had cardiovascular events.

Since measurements of the two biomarkers require only plaque and vessel wall boundaries, these biomarkers can be used to analyze carotid images acquired by other imaging modalities if the required boundaries can be segmented from the acquired images. For example, the biomarkers introduced can be used to analyze MR images, as vessel wall boundaries can be reliably segmented from black-blood MRI (Krasinski et al. 2009a) and plaque boundaries can be obtained from multiparametric MRI and simultaneous noncontrast angiography and intraplaque hemorrhage (SNAP) imaging (Zhang et al. 2019).

Mini-Dictionary of Terms

- **Carotid arteries**: A pair of arteries on the left and right sides of the neck, connecting the heart and the brain. The location of the carotid artery is superficial and easy to observe, so carotid atherosclerosis is often used as an indicator of systemic atherosclerosis in the human body.
- **Media-adventitia boundary (MAB) and lumen-intima boundary (LIB)**: The arterial wall is composed of intima, media, and adventitia. The carotid lumen is the internal space of the carotid artery. The LIB is the interface between the lumen and intima and the MAB is the interface between the media and adventitia.
- **Carotid atherosclerosis**: A major source of atherosclerotic emboli that would block cerebral arteries and thus lead to ischemic strokes. Atherosclerosis is the thickening, hardening, and loss of elasticity of the arteries.
- **Plaque texture**: The pattern in the grayscale image of the plaque, for example, contrast/smoothness/brightness. A set of 376 textural features from 9 different texture extraction techniques was generated for each plaque in the left and right arteries in this review.
- **Vessel-wall-plus-plaque thickness**: The thickness between the carotid lumen-intima boundary and the media-adventitia boundary to quantify the progression/regression of atherosclerosis.

Key Facts of 3D Carotid Ultrasound Biomarkers

- Two 3DUS biomarkers, one based on carotid plaque texture and the other based on vessel-wall-plus-plaque thickness (VWT) distribution, are able to establish the effect of pomegranate juice/tablet on carotid atherosclerosis.
- The plaque texture biomarker requires 20 times fewer subjects than total plaque volume (TPV) to detect the effect of pomegranate.
- The weighted vessel-wall-plus-plaque thickness average ($\overline{\Delta VWT}_{\text{Weighted}}$) requires 14 times fewer subjects than TPV to detect the effect of pomegranate.
- Correspondence alignment of the VWT maps leads to a 20% reduction in sample size required by $\overline{\Delta VWT}_{\text{Weighted}}$ to establish the significance of the treatment effect conferred by pomegranate intake.
- $\overline{\Delta VWT}_{\text{Weighted}}$ is less sensitive than the plaque texture biomarker, but $\overline{\Delta VWT}_{\text{Weighted}}$ measurement does not require plaque segmentation. The media-adventitia boundary (MAB) and lumen-intima boundary (LIB) required for VWT measurements can be segmented reliably by automatic algorithms.

Summary Points

- Sensitive and cost-effective biomarkers are required to evaluate the efficacy of dietary treatments, which confer a smaller effect than medical therapies.
- Pomegranate is a dietary supplement shown to inhibit LDL oxidation and attenuate atherosclerosis development in animal studies, but a recent placebo-controlled trial involving 289 subjects showed the pomegranate does not have a significant effect on intima-media thickness (IMT).
- We reviewed a carotid plaque texture biomarker and a VWT-based biomarker that can establish the effect of pomegranate based on 3DUS imaging.
- The plaque texture biomarker was generated by a workflow involving the reduction of dimensionality of 376-dimensional plaque textural features through principal component analysis (PCA) and LPP.
- VWT was measured and projected onto a 2D carotid atlas, resulting in 2D VWT maps. VMT maps obtained from different subjects were aligned using a correspondence optimization approach. The change of VWT (ΔVWT) of a subject was obtained by taking the difference between the VWT maps obtained at baseline and a follow-up scanning session. The weighted average of ΔVWT was computed by weighting the ΔVWT map with a map highlighting anatomic locations likely to exhibit plaque change.
- Future directions in carotid 3DUS biomarker development include integration of plaque textural and VWT-based features to further increase the sensitivity in treatment effect evaluation, and the use of deep learning models to analyze either the 3DUS image or the VWT maps directly.

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NMR Metabolomics for Marker Discovery of Metabolic Syndrome

31

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Abstract

The metabolic syndrome (MetS) is a multifactorial disease developed due to the accumulation and persistence of several risk factors associated with disrupted metabolism. To date, there is lack of efficient tools to evaluate the stage of MetS and its risk factors including carbohydrate dysfunction, dyslipidemia, inflammation, oxidative stress, gut microbiota dysbiosis, and anxiety. Expectantly, nuclear magnetic resonance (NMR) metabolomics has emerged as a promising source of new molecular markers due to its advantages across other metabolomic approaches. This chapter highlights the six major risk factors associated with MetS and related diseases, discussing their potential and weaknesses as biomarkers according to the current evidence available in the literature. Together, it is proposed a profile of metabolites for each risk factor obtained from NMR approaches to assess the severity of the risk factors associated to MetS.

Keywords

Biomarker · NMR · Metabolic syndrome · Metabolism deregulation · Carbohydrate dysfunction · Dyslipidemia · Inflammation · Oxidative stress · Gut microbiota dysbiosis · Anxiety

Abbreviations

1C	One carbon
8-OHdG	8-hydroxy-2'-deoxyguanosine
AAAs	Aromatic amino acids
BCAAs	Branched-chain amino acids
C3	Propionylcarnitine
CAF	Cafeteria
CRP	C-reactive protein

CVD	Cardiovascular disease
DMA	Dimethylamine
FMO3	Flavin-containing monooxygenase 3
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography coupled with mass spectrometry
GlycA	Glycoprotein acetyl
GlycB	Sialic acid
GSH	Glutathione
GSSG	Oxidized glutathione
H202	Hydrogen peroxide
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HOMA-IR	Homeostasis Model Assessment of IR
IR	Insulin resistance
LC-MS	Liquid chromatography coupled with mass spectrometry
LDL	Low-density lipoprotein
LPC	Lysophosphatidylcholine
Lp-PLA2	Lipoprotein-associated phospholipase A2
MetS	Metabolic syndrome
MS	Mass spectrometry
NCD	Noncommunicable disease
NMR	Nuclear magnetic resonance
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SFA	Saturated fatty acid
SUCNR1	Succinate receptor 1
T2D	Type 2 diabetes mellitus
TMA	Trimethylamine
TMAO	Trimethylamine oxide
VLDL	Very-low-density lipoprotein
WHO	World Health Organization

Introduction

Metabolic syndrome (MetS) is defined by the World Health Organization (WHO) as a pathologic condition characterized by abdominal obesity, insulin resistance (IR), hypertension, and hyperlipidemia (Saklayen 2018). This noncommunicable disease (NCD) has become a major health hazard of the modern world. Starting in the Western countries and with the spread of the Western lifestyle across the globe, it has become a truly global problem (Saklayen 2018). MetS is considered a multifactorial disease, which means that its development is influenced by a group of risk factors associated with disrupted metabolism (Grundy 2016). Multifactorial diseases are caused by different single factors but also by a combination of altered metabolic situations (genetic, environmental, physiological, metabolic, cellular, and molecular

elements). If these situations interact together and are extended over time, it can lead to a pathologic state (Vassallo et al. 2016).

Nowadays, there is lack of efficient tools to evaluate the stage of the MetS and their risk factors, which essentially includes carbohydrate and lipid metabolism, inflammation, oxidative stress, and gut microbiota (van Ommen et al. 2009; Hernandez-Baixauli et al. 2020). Additionally, a new risk factor that has been unnoticed and could have a deep impact on MetS is anxiety and its subsequent disorders (Tang et al. 2017). Focusing on these six risk factors, several lifestyle aspects could be modified to prevent the development of these abnormalities prior to the development and severity of MetS (i.e., diet, nutritional habits, and physical activity). Thus, including driven changes in diet and nutritional habits, also known as personalized nutrition, has become a promising tool and has acquired greater relevance in society to prevent and assess the severity of metabolic diseases (González-Peña and Brennan 2019).

The study of the metabolome provides personal information on the physiological and pathophysiological states, as well as the environment, becoming a valuable tool to profile the risk of the subjects and to evaluate the stage of the MetS. Advances in omics technologies have led to the possibility of characterizing the metabolism of every subject from a holistic point of view, opening a wide array of possibilities for phenotypic characterization and providing a more accurate health assessment that contributes to improve the quality of life (van Ommen et al. 2009). To study the metabolome, the most common techniques applied in metabolite profiling studies are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) hyphened to chromatographic techniques (Silva et al. 2020).

Nowadays, NMR studies have increased, focusing on the research of biomarkers and the metabolic profiling of MetS. The relative ease of sample preparation, the ability to quantify metabolite levels, the high level of experimental reproducibility, and the inherently nondestructive nature of NMR spectroscopy have made it the preferred platform for long-term or large-scale metabolomic studies (Edison et al. 2021). Specifically, ^{1}H-NMR spectroscopy has emerged as the most powerful NMR approach to assess the severity of diseases (Mancano et al. 2018; Silva et al. 2020). Furthermore, the most popular biological fluids used in metabolomics are plasma or serum and urine, while other fluids and tissues are not well explored yet. Those biofluids are relatively easy to collect with minimal invasive procedures and their metabolome reflects the individual changes in metabolism (Edison et al. 2021).

In this chapter, it is summarized the main biomarkers obtained by NMR metabolomics to assess the severity of the risk factors associated to MetS. (The metabolic biomarkers are discussed through six different risk factors: carbohydrate dysfunction, dyslipidemia, inflammation, oxidative stress, gut microbiota dysbiosis, and anxiety) (Fig. 1).

Carbohydrate Dysfunction

Carbohydrate metabolism disorders are dysfunctions that affect the catabolism and anabolism of several saccharides. The bulk of these dysfunctions are hereditary metabolic disorders, nevertheless it can be acquired being IR the most representative

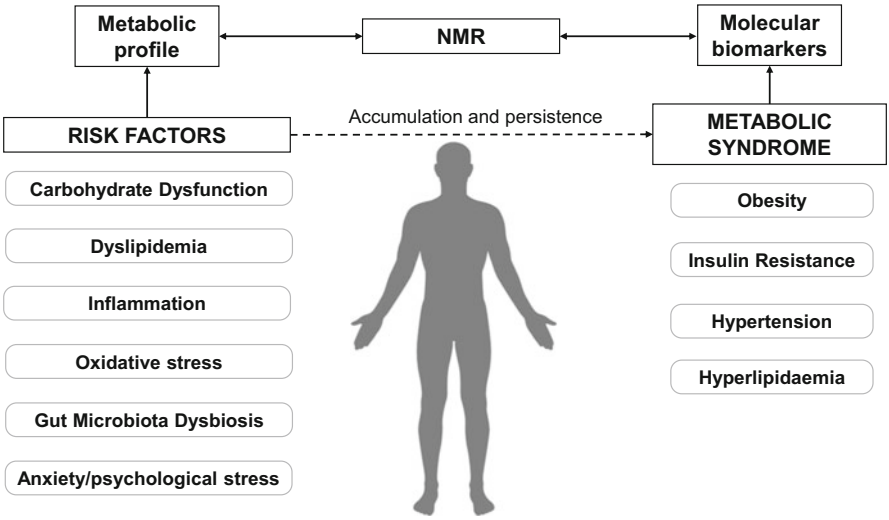

Fig. 1 This figure shows a summary of the relationship between risk factors, metabolic syndrome, and NMR metabolomics for marker discovery. The accumulation and persistence during time of different risk factors (i.e., carbohydrate dysfunction, dyslipidemia, inflammation, oxidative stress, gut microbiota dysbiosis, and anxiety) induces MetS and related diseases. The detection of these risk factors can be done by metabolic profiling and molecular markers obtained from NMR metabolomic approaches

disorder. IR has become a worrying situation for a considerable part of the population, being one of the features included in the MetS and mainly caused by altered metabolism of carbohydrates. IR can usually lead to type 2 diabetes mellitus (T2D), which accounts approximately 95% of diabetes cases worldwide, and importantly increases the risk for cardiovascular disease (CVD). This situation is currently assessed by clinical determinations such as HOMA-IR, HbA1c determination, or circulating levels of leptin and adiponectin and the ratio between both (Cosentino et al. 2020). However, these standard determinations are not useful to determine the different stages of T2D, as the assessed parameters get altered when the disease is already established. The main metabolites detected by NMR approaches include those that are involved in carbohydrate metabolism such as glucose or lactate, as well as uric acid, branched-chain amino acids (BCAAs), aromatic amino acids (AAAs), glutamine, glutamate, citrate, and propionylcarnitine. A summary of these metabolites can be found in (Table 1).

Glucose

A key metabolite in carbohydrate metabolism that can easily be detected by NMR is glucose. The ability to maintain the homeostasis of glucose is critical in carbohydrate dysfunction development. Indeed, high circulating glucose levels can lead to pancreatic β-cell alteration which, if untreated, will consequently drive to IR and T2D (Brereton et al. 2016). NMR was used in a study comparing metabolite alterations among young

Table 1 Biomarkers involved in carbohydrate dysfunction identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Plasma	Adolescents who had overeaten during childhood	Decreased: Citrate	(Hübel et al. 2021)
	Children and adolescents with or without obesity	Decreased: Glucose, glutamine	(Bervoets et al. 2018)
Serum	Normal-weight versus overweight young adults	Increased: Glucose, lactate	(Pasanta et al. 2019)
	Patients with diabetes and/or diabetes related to comorbidity, compared to healthy controls	Increased: Glucose	(Rawat et al. 2019)
	Patients with hyperuricemia and patients with gout compared to healthy controls	Increased: Glucose	(Zhang et al. 2018)
	Comparison between two Finnish cohorts: Pieksamaki and Health 2000 (6, 5 years follow-up)	Increased: Lactate Altered: BCAAs, AAAs	(Würtz et al. 2012)
	Comparison between diabetic and nondiabetic patients	Increased: Glucose, lactate, uric acid, BCAAs, AAAs	(Gogna et al. 2015)
	Comparison between obese and normal-weight twins	Increased: BCAAs, AAAs Decreased: Glutamine, citrate	(Bogl et al. 2016)
	Diabetic patients treated with glucose-lowering drugs	Increased: BCAAs	('t Hart et al. 2018)
	Pregnant women transitioning from gestational diabetes to T2D	Increased: BCAAs	(Andersson-Hall et al. 2018)
	Comparison between obese and normal-weight young adults	Increased: Glucose, lactate, BCAAs, glutamate Decreased: Glutamine	(Htun et al. 2021)
	Patients with heart failure, divided by low/intermediate/high myocardial energy expenditure	Increased: BCAAs, AAAs	(Du et al. 2014)
Urine and serum	Comparison between nonthreated and histidine-threated obese patients	Decreased: Citrate	(Du et al. 2017)

adults with either normal weight and overweight, where glucose levels were increased in serum of the last group of patients (Pasanta et al. 2019). Moreover, other findings demonstrate that serum glucose concentration is directly related to the state of T2D, which supports the determination of this metabolite as a useful tool for the diagnosis of this disease (Rawat et al. 2019). Glucose was also included as a biomarker for MetS in a study that aimed to build a biomarker signature by serum profiling with NMR (Zhang et al. 2018). Unexpectedly, a study that was performed with adolescents with or without obesity reported lower levels of circulating glucose in obese patients, suggesting this parameter might be age dependent (Bervoets et al. 2018).

Lactate

Since glucose levels can be also assessed rapidly by glucometers, which are highly available and accessible nowadays, other options should be considered to use NMR with this goal. Glucose metabolism is highly involved in lactate homeostasis, meaning alterations in carbohydrate metabolism are also reflected in lactate levels (Würtz et al. 2012). Therefore, this metabolite is another potential candidate as biomarker for diseases associated with glucose disruption, such as MetS. Indeed, altered metabolism in pancreatic β-cells results in abnormal accumulation of lactate in urine, blood, and cerebrospinal fluids (Abu Bakar et al. 2015). Different clinical studies assessed lactate levels with NMR approaches in patients with altered metabolic profile, demonstrating it was increased in serum from patients with higher risk for MetS (Würtz et al. 2012; Gogna et al. 2015; Pasanta et al. 2019).

Uric Acid

Uric acid is stated as one of the most important molecular markers for early development of MetS and other metabolic diseases, as its overproduction is related to metabolic disruptions such as IR, hypertension, or nonalcoholic fatty liver disease (NAFLD), among others (Barragan et al. 2019). Several clinical studies have shown increased serum levels of uric acid in patients suffering MetS or T2D (Cox et al. 2012). Furthermore, uric acid levels are also loaded by fructose intake, which could be useful as a predictor of further metabolic disorders, since Western diets are usually fructose rich (Johnson et al. 2018). Moreover, uric acid was elevated in T2D, as determined by NMR (Gogna et al. 2015).

BCAAs and AAAs

Circulating levels of BCAAs are also considered in metabolic assessment, as amino acids participate in the regulation of glucose, lipid, and protein synthesis, among other functions. Increased levels of BCAAs were observed in obese subjects, compared to lean, indicating increased catabolism of BCAAs and IR development in obesity. Being BCAAs easily detected and quantified by both NMR and MS methods, these metabolites also appear as potential candidates for MetS detection. On the other hand, phenylalanine and tyrosine are AAAs that can be involved in metabolic alterations. Therefore, its detection can also be used for diagnosis. Studies using NMR techniques pointed out both BCAAs and AAAs as biomarkers for MetS, obesity, and T2D. Particularly, altered levels of BCAAs are associated with disruption of carbohydrate metabolism, informing about poor glycemic control in healthy adults and in patients with prediabetes and T2D (Würtz et al. 2012; Gogna et al. 2015; Chen et al. 2016; ‘t Hart et al. 2018), and in pregnant women with gestational diabetes (Jiang et al. 2019). However, findings are controversial when choosing specific amino acids, and no concluding outcomes have been reported to date

(Du et al. 2017). Therefore, BCAAs and AAAs should be considered as whole units for metabolite determination using NMR approaches.

Glutamine and Glutamate

Other amino acid as glutamine has been associated to homeostasis of carbohydrate metabolism, showing an inverse relationship with T2D risk. Glutamine is part of the glutamate-glutamine cycle, and glutamate was reported to be directly related to glucose metabolism disruption (Tulipani et al. 2016). Moreover, glutamate levels also varied depending on weight, being higher in young obese patients than in young normal-weight patients (Htun et al. 2021). In this case, detection of both glutamine and glutamate by NMR is promising for the prevention of carbohydrate metabolism dysfunction.

Citrate

Citrate levels are also altered when carbohydrate metabolism is impaired in plasma and urine. However, data of citrate levels evaluated by NMR are scarce. Thus, in a study using NMR that compared urine citrate levels in obese women treated either with an amino acid (histidine) or placebo showed higher citrate levels in amino acid–treated obese women (Du et al. 2017). In addition, citrate plasma levels were lower in adolescents who had overeaten during childhood, which increases the probabilities to develop metabolic diseases in adulthood (Hübel et al. 2021).

Propionylcarnitine

Propionylcarnitine (C3) is a short-chain acylcarnitine that could also be included as a MetS biomarker in NMR studies. Being acylcarnitine highly related to carbohydrate and lipid metabolism regulation, its concentrations vary when these processes get altered (Zhang et al. 2014). C3 was increased in patients with IR and high risk for T2D (Bene et al. 2019), and when combined with other metabolites (BCAAs, glutamate/glutamine, or methionine). Additionally, it provides more robust data to differentiate metabolically lean from obese patients (Dorcely et al. 2017). Other results report higher C3 levels in patients with impaired glucose metabolism (Mai et al. 2013). Furthermore, considering that the different NMR approaches have lately improved acylcarnitine profile screening (Saito et al. 2017), determination of C3 as a biomarker for MetS will be more reliable.

Dyslipidemia

Dyslipidemia is one of the metabolic disruptions with higher prevalence worldwide (Hernandez-Baixauli et al. 2020), and strongly related to CVD. As carbohydrate dysfunction, dyslipidemia is one of the considered risk factors for developing MetS.

Table 2 Biomarkers involved in dyslipidemia identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Plasma	Adolescents who had overeaten during childhood	Increased: VLDL, remnant cholesterol, apoprotein B, glycoprotein acetyls, omega-6 PUFAs	(Hübel et al. 2021)
	Children and adolescents with or without obesity	Increased: Lipids	(Bervoets et al. 2018)
Serum	Normal-weight versus overweight young adults	Increased: CH3, CH2 lipids	(Pasanta et al. 2019)
	Comparison between diabetic and nondiabetic patients	Increased: Glycerol, SFAs, choline, 3-hidroxybutyrate	(Gogna et al. 2015)
	Comparison between obese and normal-weight twins	Increased: Glycoprotein, SFAs Decreased: PUFAs, large HDL-C	(Bogl et al. 2016)
	Pregnant women transitioning from gestational diabetes to T2D	Increased: 3-hydroxyisobutyrate	(Andersson-Hall et al. 2018)
	Comparison between obese and normal-weight young adults	Increased: 3-hydroxybutyrate Decreased: Choline	(Htun et al. 2021)
	A 7-year follow-up cohort from a representative population regarding changes in body weight	Increased: VLDL, small LDL, small HDL Decreased: Large-HDL-C	(Wahl et al. 2015)
	Patients with heart failure, divided by low/intermediate/high myocardial energy expenditure	Increased: 3-hydroxybutyrate, acetone, succinate	(Du et al. 2014)
Urine and serum	Comparison between nonthreated and histidine-threated obese patients	Decreased: Choline	(Du et al. 2017)

It implies lipid metabolism impairment, with an abnormal functionality of the adipose tissue, an altered lipid transport, and an increased risk for developing other metabolic dysfunctions, given the pro-inflammatory effects of these lipidic alterations. Dyslipidemia is currently assessed by clinical observations of typical biomarkers such as circulating lipid levels, glucose, and hormones (Hernandez-Baixauli et al. 2020). However, these determinations are not helpful to determine the stage of the disease and the early diagnosis, as it indicates the presence of the disease and not the severity (Hernandez-Baixauli et al. 2020). In this context, recent studies have focused on determining the metabolite profile of patients with elevated risk for MetS in the context of dyslipidemia. A summary of these metabolites can be found in Table 2.

Fatty Acids

Many studies using NMR approaches had observed different fatty acid profile among patients with metabolic disruptions. On the one hand, polyunsaturated fatty

acids (PUFAs) were decreased in obese patients compared to their normal-weight twins (Bogl et al. 2016). PUFAs are more beneficial than saturated fatty acids (SFAs), given its antioxidant and anti-inflammatory properties, among others. Therefore, these results indicate that increase in adiposity promotes an unhealthy lipid profile. PUFAs were also increased in adolescents who had overeaten during their childhood, regardless of the current weight. These highlights an established metabolic disruption that could further affect the health of these patients (Hübel et al. 2021). Moreover, increased SFAs were observed in serum of diabetic patients, compared to nondiabetic, reminding about the close relationship between carbohydrate and glucose metabolism (Gogna et al. 2015). Similarly, increased CH2 and CH3 lipids were observed in serum samples of overweight patients in a study that compared normal-weight and overweight patients. These lipids are released by lipoproteins (VLDL and LDL) in blood, and altered levels could indicate disrupted lipid metabolism (Pasanta et al. 2019).

Choline

Choline is involved in the mobilization of fat from liver, being negatively associated with hepatic fat accumulation. Its levels have been assessed in many studies using NMR, where it is suggested as another possible biomarker for detecting disruption of lipid metabolism. Hence, choline levels were diminished in serum or urine of obese patients, compared to either normal-weight adults or obese adults treated with histidine. Also, its levels were altered in a study comparing the metabolome of overweight versus normal-weight patients, but there were no significant changes between groups (Du et al. 2017; Htun et al. 2021).

Lipoproteins

Lipoproteins, key components in lipid metabolism, are indeed highly useful assessing metabolic abnormalities. These proteins indicate lipid levels in blood, including triglycerides, total cholesterol, and fatty acids. The most studied lipoproteins are chylomicrons, which are the densest ones, followed by VLDL, LDL, and HDL. The information provided by these proteins is given not only by its circulating levels but also by its size and density, showing the current functional status of lipid metabolism. Recent studies using NMR approaches demonstrated that lipoprotein characterization can be used for diagnosis of metabolic alterations, as it provides information about diet and lifestyle, regardless of weight and apparent metabolic abnormalities. In this sense, minimal alterations in lipid and cholesterol metabolisms can be detected in apparently healthy patients, which could prevent the development of other alterations included in the MetS (Wahl et al. 2015; Bogl et al. 2016; Van Duynhoven and Jacobs 2016; Hübel et al. 2021).

Ketone Bodies

Another way to assess impaired lipid metabolism would be quantifying ketone bodies, which are products of fat catabolism that are used as alternative substrates to glucose when carbohydrate intake is low and there is an excess of circulating free fatty acids. Elevation in the main ketone bodies (3-hydroxybutirate and acetone) have been detected by NMR in several studies (Du et al. 2014; Gogna et al. 2015; Andersson-Hall et al. 2018; Htun et al. 2021). For example, a clinical study about identification of metabolic phenotypes in young adults with obesity observed an increase of 3-hydroxybutirate levels in plasma (Htun et al. 2021). Recent studies observed increased levels of 3-hydroxybutirate in patients with high myocardial energy expenditure, suggesting disturbances in lipid utilization by this tissue. Indeed, 3-hydroxybutirate is capable of inhibiting β-oxidation, although the mechanism behind this inhibition remains unclear. Following these results acetone was also increased in patients with both intermediate and high myocardial energy expenditure. Consistently, these two metabolites, 3-hydroxybutirate and acetone, could be other candidates as biomarkers for early detection of MetS (Du et al. 2014).

Inflammation

Obesity and MetS are described as risk factors for inflammatory diseases. Inflammatory biomarkers from NMR profiles are useful and beneficial for the detection of metabolic alterations and inflammatory-related diseases. Mainly, the role of N-acetylglycoproteins and lysophospholipids is important in NMR analysis among other metabolites, which are more commonly analyzed by other methods (Esser et al. 2014). A summary of these metabolites can be found in Table 3.

Table 3 Biomarkers involved in inflammation status identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Plasma	Participants with and without T2DM were aged N18 years	Increased: N-acetylglycoproteins	(Dullaart et al. 2015)
	65 obese and 37 normal-weight children	Increased: N-acetyl glycoproteins and lactate Decreased: α-ketoglutarate, glucose, citrate, and chlorinated phospholipids	(Bervoets et al. 2018)
	Plasma metabolic signatures in adults with obesity and morbid obesity	Increased: Amino acids (alanine, valine, and isoleucine), hydroxibutyrate Decreased: Lysophosphatidylcholines	(Stroeve et al. 2016)

N-Acetylglycoproteins

Modifications in proteins are numerous and important for the biological function. Glycosylation is the addition of one or more chains of carbohydrates (glycans) to a protein and glycosylation is a key factor in some biological processes (Fuertes-Martín and Vallvé 2020). Protein glycosylation is composed of O-glycans, N-glycans, and glycominoglycans. N-glycosylation is mostly found in circulating proteins and O-glycosylation is predominant in protein signaling and intracellular mechanisms. In fact, N-glycosylation could be easily disturbed by pathophysiological conditions such as inflammation, thus making N-glycans as emerging powerful biomarker of inflammatory diseases (Fuertes-Martín and Vallvé 2020).

The altered synthesis of N-glycans has been reported in diverse pathophysiological studies that show changes in its circulatory concentration, which might reflect the development of different diseases, such as CVD and T2D. Therefore, human glycome has become a novel tool to identify biomarkers and potential mechanistic mediators of pathogenesis, such as glycoproteins of the cell membrane, which play a significant role in the immune response (Akinkuolie et al. 2015; Fuertes-Martín and Vallvé 2020). In a study performed by Lawler and collaborators, GlycA signal was identified by NMR as a glycoprotein-N-acetyl methyl group signature, which was associated with MetS-related diseases (Lawler et al. 2016). Only a small part of acute-phase glycoproteins contributes to this GlycA signal, since NMR technique cannot detect concentrations below to 20 μmol/L. α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin, and transferrin, which are secreted by neutrophil granules and in the liver by hepatocytes, are the major contributors of this GlycA signal (Fuertes-Martín and Vallvé 2020). GlycA identifies aggregates of glycan moieties on circulating glycoproteins, the majority of which are acute-phase reactants and immune response proteins, related to the activation of systemic inflammatory pathways and the development of IR and β-cell dysfunction (Lawler et al. 2016). Interestingly, Bervoets and collaborators studied the plasma metabolite of obese children with NMR, and obese children showed an increased N-acetyl glycoprotein signal compared to healthy children (Bervoets et al. 2018). Therefore, the activation of the hexosamine pathway, which is related with lower levels of glutamine and glucose, leads to a GlycA increase. Because of the intraindividual variability present in traditional inflammatory cytokines, GlycA emerges as a better biomarker option for systemic inflammatory response.

Sialic acid (GlycB), together with GlycA, provides information of acetyl groups of proteins bonded to N-acetylgalactosamine, N-acetylneuraminic, and N-acetylglucosamine acid. The concentration of free fraction of acetyl groups not bonded to proteins of N-acetylgalactosamine, N-acetylneuraminic, and N-acetylglucosamine are detected in the GlycF area (Serés-Noriega et al. 2021).

To conclude, glycoproteins of acute-phase reactants have been emerged as important biomarkers for the detection, prognosis, and therapeutic monitoring of acute and chronic tissue inflammation in multiple pathologies.

Lysophospholipids

Lysophospholipids are signaling molecules produced from phospholipid hydrolysis, which transport free fatty acids, choline, and phosphatidylglycerol through different tissues (Bas et al. 2016). The hydrophobic tail and the hydrophilic head group determine their specific biological function. Their signaling action is related to inflammation, insulin production, and insulin sensitivity through the interaction of lysophospholipids with G protein–coupled receptors. Thereby, lysophospholipids might be essential molecules in obesity and related disorders such as NAFLD, steatohepatitis, T2D, and MetS (Bas et al. 2016). Some lysophospholipids species, such as lysophosphatidilcholines (LPCs), were altered in the plasma of obese individuals. Lipoprotein-associated phospholipase A2 (Lp-PLA2), which is defined as an inflammatory marker, and lecithin enzymatic activity synthetize significant levels of circulatory LPCs under inflammatory conditions. Increased levels of LPCs trigger the release of second messengers related to G protein–coupled receptors (Alkan and Mungan 2018). In a study of obesity, LPCs concentrations were inversely correlated with increased C-reactive protein (CRP) (Bas et al. 2016). Therefore, LPCs might be an important biomarker to detect inflammatory states associated with MetS and other related disorders.

Oxidative Stress

Oxidative stress, a notable risk factor for several diseases, comes out when an imbalance of homeostasis between oxidant and antioxidant agents appears. Reactive oxygen species (ROS) are the main oxidant agents produced by the aerobic organism in normal metabolic processes (lipid metabolism, cellular respiration, antibacterial defense, etc.) and external exposures (ionizing radiation, smoking, toxins, etc.) (Pietzner et al. 2017). The development of pathological conditions such as MetS triggers with the presence of increasing oxidative stress (Hernandez-Baixauli et al. 2020). Thus, it is essential to find different metabolites monitoring oxidative stress that might be potential biomarkers of MetS determined by NMR metabolomics. A summary of these metabolites can be found in Table 4.

Uric Acid and Allantoin

Uric acid is known as the major antioxidant in plasma that accounts for as much as two-thirds of the total antioxidant capacity of plasma. Uric acid is determined as the final oxidation product of purine catabolism in humans. High levels of circulatory uric acid could mean the body is protecting itself from effects of oxidant agents with endogenous antioxidants (Sidorov et al. 2019). Despite being the major antioxidant agent in plasma, a duality of the uric acid as biomarker exists, being an antioxidant signal in plasma and pro-oxidant signal within the cells (Hernandez-Baixauli et al. 2020). Considering this feature, allantoin is proposed as an alternative biomarker,

Table 4 Biomarkers involved in oxidative stress processes identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Serum	Clinical student with 35 men and 47 women, aged 30–60 years with MetS	Increased: Sphingolipids Decreased: Serine and glycine	(Palmnäs et al. 2018)
Urine	A metabolomic comparison of urinary changes in type 2 diabetes in human	Increased: Creatinine, N-acetyl groups (including glycoproteins), N-methylnicotinate (NMN acid), aminohippurate, hippurate, PAG, allantoin, fumarate, and succinate Decreased: Creatinine, N-acetyl groups, N-methylnicotinate, aminohippurate, hippurate, PAG, allantoin, fumarate, and succinate	(Salek et al. 2007)
	4680 men and women ages 40–59 years from 17 population samples in four countries	Increased: Pseudouridine	(Elliott et al. 2015)

being the final product of uric acid oxidation from purine metabolism and working as a pro-oxidative agent (Abdul Ghani et al. 2019).

Allantoin production is activated due to increased levels of ROS in the body, upregulating nonenzymatic processes. Urinary allantoin has been determined in several preclinical studies, highlighting its stability over different storage conditions as an oxidant biomarker and stand out among other metabolites in MetS and related diseases such as atherosclerosis, T2D, and obesity (Pelantová et al. 2016; Abu Bakar Sajak et al. 2017; Abdul Ghani et al. 2019; Guirro et al. 2020). This finding was also found in obese CAF (cafeteria)-fed rats where allantoin was increased (Guirro et al. 2020). Nevertheless, in an urinary metabolomics study, allantoin is decreased in rodent models and in human subjects with T2D, while allantoin is increased in obese rats. These findings suggest that decreased urinary allantoin levels indicate reduced glomerular filtration rate, as allantoin is not reabsorbed across the proximal tubule. Thereby, its urinary concentration is thought to be related to glomerular filtration (Salek et al. 2007; Abu Bakar Sajak et al. 2017). However, more studies in human through NMR analysis are necessary to confirm the evidence obtained in several preclinical studies.

8-OHdG and Pseudouridine

8-hydroxy-2′-deoxyguanosine (8-OHdG) and pseudouridine are urinary noninvasive markers for oxidative stress related to metabolic and energy impairment. The first one is a typical biomarker that represents oxidative stress levels in DNA, whereas pseudouridine is originated from ribosomal and transfer RNA degradation reflecting RNA turnover (Guirro et al. 2020; Hernandez-Baixauli et al. 2020). Pseudouridine is an isomer of the nucleoside uridine in which uracil is attached via a carbon-carbon instead of a nitrogen-carbon glycosidic bond. Promising NMR

metabolic approaches determining pseudouridine could be led by DNA oxidative stress. In a preclinical study, pseudouridine urinary levels were elevated in CAF-fed rats compared to control group. Pseudouridine decreased in hesperidin treated group, concluding that pseudouridine may be increased in metabolic diseases, acting as an oxidative risk factor (Guirro et al. 2020). In a clinical study with an urinary NMR metabolomics analysis, pseudouridine was positively correlated with body mass index (BMI), reflecting an increased whole-body nucleic acid turnover, consistent with linked adipose tissue replacement associated with obesity (Elliott et al. 2015).

One-Carbon Metabolism Intermediates: GSH/GSSG Ratio, Glycine, and Serine

One-carbon (1C) metabolism, which supports several physiological processes such as biosynthesis, amino acid homeostasis, epigenetic maintenance, and redox defense, is associated with overweight, obesity, and metabolic-related diseases. This 1C metabolism implies a source of potential biomarkers that play a role on the transfer of one-carbon group (Ducker and Rabinowitz 2017; Hernandez-Baixauli et al. 2020). Some of the important potential biomarkers are glutathione (GSH)/oxidized glutathione (GSSG) ratio, glycine, and serine, among other precursors or intermediates of glutathione production. Nevertheless, a handicap that should be considered is the perception of oxidized metabolites to detect biomarkers of oxidative stress, since redox reactions may change the state of the metabolite (oxidized/reduced) at the time of its manipulation and interfere with the determination of the redox ratio (Ducker and Rabinowitz 2017). GSH fights against oxidative injury by reducing hydrogen peroxide (H_2O_2). Impairment of GSH production could lead to increased levels of ROS, principal authors of oxidative stress. GSH total quantification could be determined but the redox ratio calculation is highly complex. Thus, the GSH/GSSG ratio is analyzed as a potential indicator of cellular health, as it is mainly composed by reduced GSH, constituting up to 98% of cellular GSH under normal conditions (Luc et al. 2019). Other alternative metabolites that do not present problems in redox ratio determinations are glycine and serine, key precursors of GSH and intermediates of 1C metabolism. Glycine is a low molecular weight amino acid that incorporates a hydrogen atom as a sidechain. It can be considered as a potential biomarker since its chronic deficiency may affect health status. Hence, glycine levels were negatively associated with IR when measured with HOMA-IR score (Ducker and Rabinowitz 2017). Therefore, apart from being a recognized precursor of GSH synthesis, glycine also plays a role in multiple pathways, which makes it unstable as an oxidative stress biomarker, because its relationship with IR could suggest glycine as a T2D biomarker. Serine, which is a glycine derivate, was also related to IR and increased risk of T2D. Exogenous serine is metabolized to make 1C units in a condition of mitochondrial function loss, whereas extracellular glycine is needed to make GSH (Ducker and Rabinowitz 2017). 1C metabolism metabolites, which are related to oxidative stress, were highlighted in a clinical study. Serum glycine and serine analyzed by NMR metabolomics were found in

lower concentrations in a study with adults aged 30–60 years with more MetS risk factors and greater adiposity in comparison with healthy subjects (Palmnäs et al. 2018).

Gut Microbiota Dysbiosis

Human health is directly connected to gut microbiota state, relying on a symbiotic relationship between human cells and the gut microbiota. Gut microbiota composition is a key factor in different pathologies and numerous studies have determined that gut microbiota composition differs between lean and obese individuals (Quesada-Vázquez et al. 2020). Changes in diet, environmental factors, or lifestyle can cause gut microbiota dysbiosis, leading to bacterial translocation and endotoxemia, which is related to decreased number of intestinal species that play important roles in intestinal barrier permeability and proliferation of specific harmful species (Quesada-Vázquez et al. 2020). Dysbiotic microbiota releases specific metabolites that are further spread into the blood, urine, or feces, meaning these metabolites can be detected and used as biomarkers. A summary of these metabolites can be found in Table 5.

Lactate

An interesting end product of bacteria fermentation, which is indeed used as a biomarker in several biochemical processes where it plays an independent role, is

Table 5 Biomarkers involved in gut microbiota dysbiosis identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Plasma	Cross-sectional and prospective cohorts of Caucasian Spanish subjects with obesity and T2D	Increased: Succinate	(Serena et al. 2018)
	Plasma from two clinical studies from subjects with CVD risk	Increased: TMAO, choline, and betaine	(Wang et al. 2011)
Serum	Relationships between gut microbiota, plasma metabolites, and metabolic syndrome traits in the METSIM cohort	Increased: Saturated and monounsaturated fatty acids, BCAAs Decreased: Unsaturated and polyunsaturated fatty acids, glutamine Altered: Acetate, SCFAs	(Org et al. 2017)
	39 adult participants with biopsy-confirmed NAFLD and healthy donors	Increased: 2-hydroxybutyrate, lactate, and acetate	(Da Silva et al. 2018)
Urinary	Subjects aged between 26 and 61 years diagnosed with MetS	Increased: TMA, DMA, and succinate	(Sobolev et al. 2019)

lactate, produced by lactic acid bacteria, mainly *Lactobacillus* and *Bifidobacterium* (Adeva-Andany et al. 2014). Lactate is produced from the carbohydrate fermentation and participates as an intermediate metabolite such as succinate. Furthermore, lactate contributes to the maintenance of gut microbiota diversity and the synthesis of most short-chain fatty acids (SCFAs) (Adeva-Andany et al. 2014). Lactate is synthetized by several bacterial species and is metabolized to butyrate and propionate, even though lactate is not accumulated in colon of healthy subjects. Depending on bacterial species, the amount and type of products may vary. If there was a reduction of the number of bacteria that metabolize lactate, an accumulation of this product would occur in colon due to its low capacity of lactate absorption (Adeva-Andany et al. 2014). Consequently, colonic pH would be lowered, inhibiting the activity of microorganisms that metabolize lactate, for example, propionate-producing or butyrate-producing bacteria. A potentially toxic accumulation of lactate could be prevented by butyrate, which is an acetate synthesis inhibitor and the main energy source for colonocytes (Gomes et al. 2015). MetS is a well-known risk factor of other diseases like NAFLD. A clinical study with biopsy-confirmed NAFLD subjects showed high levels of lactate in comparison with control subjects. These increased levels of lactate were associated with reduced abundance of several bacterial species (*Ruminococcus, Coprococcus,* and *F. prausnitzii*) negatively related to MetS (Da Silva et al. 2018). Besides, in the presence of trimethylamine N-oxide (TMAO), lactic acid bacteria produced more lactate (Hoyles et al. 2018).

Acetate

The three most common SCFAs are butyrate, propionate, and acetate. Acetate is produced through intestinal microbial fermentation of dietary fibers in the colon, and it is described as a signaling ligand from the gut microbiome to the host metabolism at different levels (Kim et al. 2019). Acetate participates in different metabolic pathways, such as human energy balance, cholesterol synthesis, or fat accumulation in adipocytes, and it has an important role in lipogenesis. Acetate increases energy expenditure and fat oxidation affecting substrate metabolism and host energy (Hernandez-Baixauli et al. 2020). BCAAs are produced and further metabolized via cross-feeding mechanisms, altering gut integrity and impairing insulin sensitivity (Hernández et al. 2019). Acetate is positively related to *Firmicutes.* In this context, dysbiosis in the gut of obese rats increases the amount of *Firmicutes* species, increasing plasma acetate levels. This is further related to insulin action in morbidly obese individuals (Hernández et al. 2019). Moreover, when acetate levels increase, fat cells release leptin. Most of the SCFAs are oxidized and excreted in the lungs, and less than 0.005% is excreted through urine. Thus, acetate is principally analyzed in blood and feces. In a clinical study with 34 morbidly obese women and men analyzed by NMR, a positive correlation was found between increased levels of acetate and elevated levels of *Firmicutes,* and a negative correlation was found with HOMA-IR and fasting TG (Hernández et al. 2019). Besides, in a clinical study with 531 Finnish men, acetate levels were positively correlated with high bacterial

richness, low BMI and TGs, and higher HDL levels, which are negatively associated with inflammation (Org et al. 2017).

Succinate

Succinate is the major intermediary in the citric acid cycle, being situated between succinyl-CoA and fumarate in the carbohydrate metabolism, where it plays a role in propionate synthesis. It is produced both in the human body and in the gut microbiome (Zhang et al. 2008). Succinate levels were increased in several metabolic diseases such as T2D, ischemic heart disease, or hypertension, but also in obesity, concomitantly related to impaired glucose metabolism (Hernandez-Baixauli et al. 2020). Specific metagenomic signatures are associated with carbohydrate metabolism and energy production, which are linked to alterations in circulating succinate levels. Succinate has an antilipolytic function in the adipose tissue through the succinate receptor 1 (SUCNR1) (Serena et al. 2018). There it blocks adipocytes from releasing free fatty acids, which is highly important in diseases such as obesity and cardiovascular diseases. In a clinical study with a cohort of 91 subjects stratified according to obesity or T2D, a relation between microbial community, gene composition, metabolism, and circulating succinate levels was found. Circulating succinate levels were analyzed by NRM and LC-MS and were significantly higher in obese individuals compared to lean subjects. Succinate was positively associated with BMI, glucose, insulin, TG, and HOMA-IR (Serena et al. 2018). In gut microbiota, circulating levels of succinate were associated with specific changes in gut microbiota species. Particularly, increased succinate levels were positively associated to higher abundance of *Prevotellaceae* and *Veillonellaceae*, succinate-producing bacteria, and negatively associated with *Odoribacteraceae* and *Clostridaceae*, succinate-consuming bacteria, in obese subjects. Patients with elevated circulatory levels of succinate presented a significant increase of glycemia related to changes in gut microbiota, which are associated to higher barrier permeability (Serena et al. 2018). Therefore, succinate, as a microbiota-derived metabolite, has an important role in obesity and metabolic-associated cardiovascular diseases (Serena et al. 2018).

TMA, TMAO, and DMA

Trimethylamine (TMA) and TMAO are products from dietary choline and carnitine through the action of gut microbes. Choline deficiency may cause gut dysbiosis and, indeed, this metabolite is modulated by gut microbiota, which activates the conversion of dietary choline to TMA and therefore choline bioavailability is lowered and phosphatidylcholine synthesis is reduced (Hernández-Alonso et al. 2017; Quesada-Vázquez et al. 2020). TMA is further released in the liver and is metabolized to TMAO through the enzymatic activity of flavin-containing monooxygenase 3 (FMO3) (Hernández-Alonso et al. 2017). TMA and TMAO have been described

as important factors in the development of metabolic diseases, impairing glucose metabolism in the liver and inducing obesity. Hence, FMO3 expression was increased in the liver in obese and diabetic patients (Miao et al. 2015). Thus, it triggers inflammation in the adipose tissue, where it affects lipid absorption and cholesterol homeostasis. Gut microbiota has a significant role in TMA production, which is evidenced in rodent studies with germ-free mice that do not excrete TMA (Quesada-Vázquez et al. 2020). In a human-derived gut bacteria study analyzed by NMR, *Enterobacteriaceae*, which is the main TMA producer, is decreased by the presence of TMAO, what is likely to happen in the intestine (Hoyles et al. 2018).

TMAO has been described as a biomarker of some metabolic diseases such as NAFLD, IR, and CVD, both in plasma and urine (Lin et al. 2016; Quesada-Vázquez et al. 2020). Dietary perturbations and intestinal microbiota may play an important role in the variation of TMAO levels. Several studies point TMAO levels as an emerging target for therapeutic interventions and, because of that, the importance of diet and microbiota homeostasis is reinforced for cardio-metabolic health maintenance (Wang et al. 2011; Quesada-Vázquez et al. 2020). It was observed that dietary supplementation with TMAO induced atherosclerotic CVD in human, which suggests that high TMAO levels elevate CVD risk (Wang et al. 2011). More than 500 Finish men with MetS were assessed in a clinical study where human serum was analyzed by NMR, and the results showed a positive correlation between intestinal microbiota *Prevotella* and *Peptococcaceae,* and circulatory TMAO levels. On the other hand, a negative correlation was observed between *Faecalibacterium praisnitzii* and plasma TMAO levels. These correlations are described as dysbiotic and are found in human disorders, such as obesity or diabetes (Wang et al. 2011).

Dimethylamine (DMA) is also a metabolite from the dietary choline metabolism, generated from TMA absorption in the liver (Hernández-Alonso et al. 2017). Elevated blood and urine DMA levels were related to high-fat-diet-induced IR, T2D, and NAFLD in mice. In contrast, DMA urinary levels were negatively correlated to fat accumulation (Hernández-Alonso et al. 2017). Therefore, DMA, that is produced by the gut microbiota, could be described as a potential prospective biomarker that indicates increased body fat, inducing obesity and being further converted to TMAO by the liver. Hoyles and collaborators observed that DMA production was positively correlated with *Enterobacteriaceae* concentrations (Hoyles et al. 2018). In a clinical pilot study with NMR, assessing subjects with MetS, urinary levels of DMA and TMA were lower before treatment with blueberry-based meals, suggesting that blueberry polyphenols can increase DMA and TMA secretion (Sobolev et al. 2019).

Anxiety, Psychological Stress, and Related Disorders

Anxiety, psychological stress, and related conditions are emergent risk factors that have been acquiring more importance in the context of MetS (Tang et al. 2017). Anxiety disorders are complex conditions including genetic, neurological, neurochemical, and psychological factors involved in their development. The diagnosis and detection of these disorders is based on a symptom checklist, and there is a need

Table 6 Biomarkers involved in anxiety, psychological stress, and related disorders, identified in clinical studies

Biofluid	Study/Model	Metabolite	References
Plasma	Netherlands study for depression and anxiety (NESDA)	Increased: GlycA Decreased: Omega-3 fatty acids	(de Kluiver et al. 2021)
	The Brazilian longitudinal study of adult health (ELSA-Brasil)	Increased: GycA	(Brunoni et al. 2020)
	Bipolar depression and healthy control participants	Increased: Lactate Decreased: Glucose, TMAO, and GlycA	(Ren et al. 2020)
	Psychological suboptimal health status	Increased: Glutamine, GlycA, TMAO, citrate, tyrosine, and phenylalanine Decreased: Valine, isoleucine, and glucose	(Tian et al. 2016)
Serum	Anxiety related to anorexia nervosa	Increased: Glutamine Decreased: Threonine, methanol, glucose, and glycoproteins	(Salehi et al. 2021)
	Environmental stress on subjects of sea voyage and Antarctic stay	Increased: Ketone bodies (3-hydoxybutyrate and acetone), glucose, arginine, BCAA, phosphoric acid, and D-galactose Decreased: Lactate and choline	(Yadav et al. 2014)

to complement the current diagnose with objective laboratory analyses. For this reason, efforts have been made to differentiate healthy from anxious subjects by the analysis of NMR metabolomics (Table 6). Having considered the complex conditions of anxiety to elucidate the affected pathways and to identify possible biomarkers, several animal studies using brain tissue samples were conducted to serve as a model for human anxiety (Humer et al. 2020). To date, the metabolites related to anxiety disorders seem to be involved in oxidative stress, alteration in lipid and energy metabolism, and neurotransmission (i.e., glutamate-glutamine cycle or GABA metabolism) (Humer et al. 2020a). Additionally, metabolites targeting poor metabolic health might serve as distal biomarkers for anxiety (Humer et al. 2020a). To date, there are few studies profiling anxiety in MetS trying to discern between health status and diseases. Nevertheless, interesting studies focused on anxiety could lead to potential candidate biomarkers in NMR approaches (Table 6).

Lipids

Overall, many early anxiety metabolomics studies have focused on lipids (lipidomics) based on the existing connection between lipids and neuronal signaling disease (Tracey et al. 2018). Indeed, the brain is particularly enriched with PUFAs, represented by omega-6 and omega-3 fatty acids. In a cohort of depressive and anxious subjects, the decrease of omega-3 fatty acids in plasma is proposed as a

potential biomarker of anxiety (de Kluiver et al. 2021), taking into account that omega-3 has a neuroprotective effect (Larrieu and Layé 2018).

GlycA

Accumulating evidence situates GlycA, a robust inflammatory biomarker, as a biomarker for anxiety and depression, and may drive to associate anxiety with systemic inflammation (Tian et al. 2016; Brunoni et al. 2020; Ren et al. 2020; de Kluiver et al. 2021). However, the association between anxiety and inflammation markers seem to differ regarding gender and age, which might also contribute to the association of lipid metabolism and inflammation with anxiety symptoms.

Glutamine

Glutamine, as the most abundant amino acid circulating in blood, is not only essential as a neurotransmitter but also is a precursor for other neurotransmitters as glutamate and GABA, and it may be considered as a biomarker for anxiety and depression (Chen et al. 2019). Other amino acids have been detected by NMR in anxious subjects (i.e., tyrosine, phenylalanine, BCAAs, arginine, choline, etc.), but glutamine stands out among other amino acids because it shows the effect on the disruption of glutamate-glutamine cycle which is suggested to be involved in different forms of anxieties and it is easily and abundantly detected by NMR (Tian et al. 2016; Salehi et al. 2021).

Glucose

The classical determination of glucose metabolism has been extensively accepted as a biomarker for MetS, as a characteristic of the disruption of carbohydrates previously mentioned. Furthermore, altered glucose levels are found in neurological disorders such as bipolar depression (Ren et al. 2020), psychological suboptimal health status (Tian et al. 2016), anxiety related to anorexia nervosa (Salehi et al. 2021), or environmental stress (Yadav et al. 2014). The commonly assessed factor is the alteration of glucose levels, although it is highly fluctuating showing higher and lower values in contrast to healthy subjects, which is a disadvantage for being a biomarker. For this reason, this metabolite may be considered a key factor for anxiety as well as a biomarker, considering other metabolites to elucidate the profile of the risk factor.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, a summary of potential NMR metabolic biomarkers was reviewed based on different risk factors associated to the MetS. It is possible that these biomarkers may be used clinically to investigate prognosis in patients. The

evaluation of these risk factors provides a valuable tool for personalized nutrition; thus, an increasing number of nutritionists integrate metabolomics into their study design, becoming one of the most promising avenues for improving personalized nutrition (nutrimetabolomics) (Ulaszewska et al. 2019).

Having discussed the application of NMR metabolomics for the discovery of MetS biomarkers, there is a huge opportunity to develop this methodology to obtain prognostic biomarkers related to other NCD. An example is the case of cancer diseases; many studies showed impressive associations between biofluid metabolomics and cancer progression, and suggest that NMR metabolomics can be used to provide information with prognostic or predictive value (Giskeødegård et al. 2018). Other example is the case of neurodegenerative diseases, accurate and sensitive salivary biomarkers were discovered for the early diagnosis of Alzheimer's disease in a pilot study with NMR metabolomics (Yilmaz et al. 2017). In the context of noninvasive alternative biofluids, together with saliva, fecal content is a promising biofluid to discover new biomarkers, as it has been determined in NAFLD studies (Da Silva et al. 2018).

However, the translation of these findings to clinical practice is currently hindered by a lack of validation, difficulties in biological interpretation, and nonstandardized analytical procedures. In this sense, there is a need to overcome these difficulties to finally transfer those promising biomarkers to the clinical practice.

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Mini-Dictionary of Terms

- ***Fatty acid:*** *A carboxylic acid consisting of a hydrocarbon chain and a terminal carboxyl group, especially any of those occurring as esters in fats and oils.*
- ***Glycome:*** *The entire complement of sugars, whether free or present in more complex molecules of an organism.*
- ***Lipidomics:*** *The study of the structure and function of the complete set of lipids (the lipidome) produced in each cell or organism as well as their interactions with other lipids, proteins, and metabolites.*

- ***Microbiome:*** *The microbiome comprises all the genetic material within a microbiota (the entire collection of microorganisms in a specific niche, such as the human gut). This can also be referred to as the metagenome of the microbiota.*
- ***Neurotransmitter:*** *A chemical substance which is released at the end of a nerve fiber by the arrival of a nerve impulse, and by diffusing across the synapse or junction, effects the transfer of the impulse to another nerve fiber, a muscle fiber, or some other structure.*
- ***Nutrimetabolomics:*** *The implementation of metabolomics tools in the nutritional sciences has been used to identifying metabolic diseases influenced or modulated by the food metabolome.*

Key Facts of Metabolic Syndrome

- The global prevalence of MetS can be estimated to be around 20.6% of the population, with men having greater prevalence than women (24.9% vs. 18.3%).
- Westernized (unhealthy) dietary patterns like overnutrition and sedentary lifestyle may trigger MetS development.
- Characteristic features of MetS are abdominal obesity, insulin resistance (IR), hypertension, and hyperlipidemia.
- Detection of metabolomic signature of MetS could be a key factor to evaluate, prevent, or treat this disease.
- Lifestyle modification and weight loss should be at the core of treating and preventing the MetS.

Key Facts of NMR Metabolomics

- By the early 2010s, while discoveries via metabolomics had not yet been translated into accepted diagnostic tests or treatments, the scientific value of metabolomics in mechanistic biochemistry was becoming clear.
- Metabolomics has strong possibilities in different biological areas because it provides an integrated picture of genomic, transcriptomic, and proteomic variation.
- Over the last years, NMR has emerged as one of the three principal analytical techniques used in metabolomics (being the other two GC-MS and LC-MS).
- The NMR is a rapid and reproducible technique that preserves the integrity of metabolites and specimens, allowing them to be investigated subsequently by other means.
- However, NMR presents lower limits of detection typically being 10–100 times less sensitive than other analytical techniques, including both GC-MS and LC-MS.

Summary Points

- Metabolic syndrome is a multifactorial disease developed due to the accumulation and persistence of several risk factors.

- It is important to evaluate the stage of the risk factors to target metabolic syndrome.
- The risk factors considered are carbohydrate dysfunction, dyslipidemia, inflammation, oxidative stress, gut microbiota dysbiosis, and anxiety.
- NMR is a promising source of metabolic markers.
- A metabolic profile of markers is important to assess the stage of MetS.

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Central and Peripheral Biomarkers for the Study of Appetite Regulation in Humans

32

Juan José Hernández Morante and Carlos Manuel Martínez

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Abstract

The largest global epidemic in the twenty-first century is obesity, since more than 700 million adults had obesity in 2015. Obesity is a consequence of an energy intake greater than energy expenditure. But under this apparently obvious sentence, there is a huge factor network involved in maintaining a balance between

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human eating episodes and energy requirements. Evidently, nutrition is a basic human need, and therefore, there is a narrow physiological control of hunger and satiety mainly performed by neuronal and hormonal factors. The problem is that, in addition to these internal factors, there is a large extent of external (social, psychological, educational) factors that may determine food intake. Unfortunately, these factors often outweigh internal factors, leading to overweight and obesity. Therefore, to carry out preventative and treatment options, it is necessary a great knowledge about appetite regulation in humans. Through this chapter, it is intended to present a framework that serves as a starting point for all those interested in the study of appetite regulation in humans.

Keywords

Neurohormonal factors · Hypothalamus · Gut hormones · Neuroimaging techniques · Appetite

Abbreviations

AEA	Anandamide
AgRP	Agouti-related peptide
ARC	Arcuate nucleus of the hypothalamus
ASL	Arterial spin labelling
ATP	Adenosine triphosphate
BOLD	Blood oxygen level-dependent signal
CART	Cocaine and amphetamine related transcript
CB1	Cannabinoid receptor 1
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CT	Computed tomography
EEG	Electroencephalography
FFA	Free fatty acids
fMRI	Functional magnetic resonance imaging
GLP1	Glucagon-like peptide 1
LFPQ	Leeds Food Preference Questionnaire
MC3	Melanocortin receptor 3
MC4	Melanocortin receptor 4
MCH	Melanin concentrating hormone
MEG	Magnetoencephalography
NE	Norepinephrine
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
OEA	Oleoylethanolamide
OXA	Orexin A
OXB	Orexin B
PEA	Palmitoylethanolamide

PET	Positron Emission Tomography
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PYY	Peptide YY
rCBF	Regional cerebral blood flow
SPECT	Single positron emission computed tomography
T3	Triiodothyronine
T4	Thyroxine
VAS	Visual analogic scales
α-MSH	α-melanocyte stimulating hormone

Introduction

The feeding process begins with the need for obtain the necessary energy supply for the body's daily maintenance. Obviously, feeding is a primary need, at the same level that the need for air, shelter, sleep, reproduction, etc. (Kenrick et al. 2010). From a physiological point of view, the first step of the feeding process involves a series of biochemical and physical signals that are transmitted to the brain, which in turn translates it into identifiable signals by the living organism in the need to feed (Berthoud 2012). These sensations are included under the concept of "hunger," and the desire to eat to satisfy this hunger is known as "appetite."

Appetite is, therefore, a sensation (a desire) that prompts the individual to satisfy it, obtaining a reward (pleasure) when he/she eats. Thus, for example, when an individual is hungry perceives a sensation, but when this individual can choose between a grilled steak, a chocolate doughnut or a salad to satisfy the hunger. If asked the reasons for making such a choice, and even if the salad has the same or even more nutrients than the steak or chocolate, the individual would adduce a set of organoleptic reasons (that is, how the food is presented: smell, color, appearance) or cultural (in their social environment they are more used to eating meat than vegetables) that justify their choice.

The most striking thing about this sensation is that an individual can feel appetite even after having satisfied their hunger (the so-called snacking, or eating between meals), a paradox that breaks the balance of the body's energy balance (balance between what that is eaten and the energy that the body expends) and that is usually the origin of the problems of overweight or obesity (Schlundt et al. 1990). Thus, appetite is a knowledge that is learned, and that is determined by our experience in the selection and preference of food and that, once satisfied, induces the release of neuronal mediators causing a pleasant sensation (Ribeiro et al. 2021).

From these concepts, there are two issues that can be questioned: why do we eat? And how is the feeling of hunger regulated? The answer to the first question is simple: we eat to live, to achieve an energy balance based on our daily energy needs. The answer to the second question, however, is much more complex, since endogenous (endocrine and metabolic), psychobiological (sensory stimuli), psychological (memory), and even cultural (eating habits depending on the region) factors intervene in this regulation (Crooks et al. 2021) (Fig. 1).

Fig. 1 Scheme of the interaction between internal and external factors (image: Freepik.com)

Although, from a physiological perspective, all these factors operate simultaneously, for a better understanding, appetite regulatory factors are usually studied separately, studying biomarkers of appetite regulation synthesized by peripheral organs, among which are leptin and ghrelin, and factors secreted by the central nervous system, such as neuropeptide Y, pro-opiomelanocortin, etc., which will be discussed later.

In summary, appetite regulation is one of the basic physiological processes of the human being. Feeding is essential to live, but an excess of feeding can also be harmful, so people constantly move in a continuous cycle of hunger/satiety that will determine when, how much, how and what to eat. Today, in our society there is a clear example of the failure of the regulation of this process, as reflected in the high number of individuals with overweight/obesity (World Health Organization 2020). This situation leads to the next question, is it that more than half of the population eats more than needed? The answer seems obvious, is that it does. But evidently, the reasons why this alarming situation is happening are not so clear, and the cause is attributed to multifactorial reasons. Therefore, some basic concepts of human appetite regulation will be revised in this chapter. Several reflections on the importance of studying appetite regulation to some common clinical situations like obesity will also be discussed.

Central and Peripheral Appetite Regulation in Humans

The multiple signals that set up the neuroendocrine system that regulates food intake in humans has two origins, central nervous system and other peripheral organs, mainly those implied in digestion and energy storage (Sumithran et al. 2011). Regarding central regulation, there are two large brain areas of interest: the hypothalamus and the brain stem. The hypothalamus is a small area located below the thalamus, in the center of the brain, and is considered the regulating center for hunger and satiety. It is structured in various neuronal nuclei that form an intricate

network of nerve pathways: the arcuate, paraventricular, ventromedial and dorsomedial nuclei, and the lateral hypothalamic area (Valassi et al. 2008; Wang et al. 2020).

The arcuate nucleus (ARN) is anatomically located at the level of the median eminence, an area characterized to be blood-brain barrier permeable, so that all signals of peripheral origin can easily access this nucleus (Parkinson et al. 2009). In fact, this area of the hypothalamus is considered the receptor area for peripheral metabolic signals, and its neurons are connected to other hypothalamic areas. The paraventricular nucleus is related to fat metabolism, the ventromedial nucleus in regulating glycemia and satiety, while the lateral hypothalamic area regulates the sensation of hunger. On the other hand, the nucleus of the solitary tract, located in the brain stem, is a region that regulates the duration of ingestion, and has multiple nerve pathways that connect with the hypothalamus, completing the central regulatory circuit (Näslund and Hellström 2007; Scott et al. 2011) (Fig. 2).

Generally, peripheral signals are received from the gut and integrated in the CNS primarily in the hypothalamus and brainstem stem, which develop an accurate orexigenic or anorexigenic response through the release of the appropriate neurotransmitters (Yu and Kim 2012).

In the ARN there are several neuron subpopulations which seem to be regulated by peripheral (gastrointestinal) appetite regulation signals: in one hand, there are the arcuate neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons, which stimulate food intake by releasing this peptides in response to circulating orexigenic ghrelin, and therefore signaling the meal initiation (Ellacott et al. 2006; Parkinson et al. 2009) or be inhibited by the induction of leptin, which induce anorexigenic stimulus, inhibition of food intake, and weight loss (Van Den Top et al. 2004). In the other hand, there are other subpopulations of neurons that can release pro-opiomelanocortin (POMC), a precursor molecule of all melanocortin derived-peptides (Wardlaw 2001), and some precursor of anorexigenic molecules (like

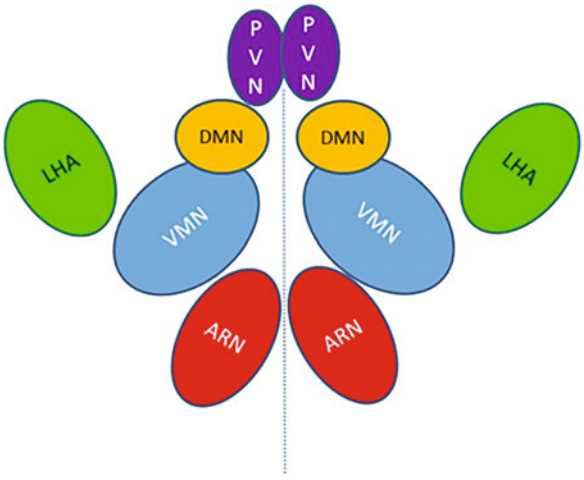

Fig. 2 Schematic representation of the main hypothalamic areas involved in appetite regulation. *ARN* Arcuate Nucleus, *VMN* Ventromedial Nucleus, *DMN* Dorsomedial nucleus, *PVN* Paraventricular Nucleus, *LHA* Lateral hypothalamus, Further information is described in the text. Dotted line represents third ventricle

α-melanocyte-stimulating hormone (α-MSH) or cocaine-and amphetamine regulated transcript (CART)) (Cone 2005). These two neuron subpopulations are not only present in the ARN, but also are projected within the lateral and ventromedial hypothalamus and the supraoptic and the paraventricular nucleus. Additionally, both subpopulations are also interconnected between them. Thus, AgRP neurons can develop a regulatory (inhibition) stimulus on POMC neurons (Lau and Herzog 2014).

By the other hand, the brainstem acts as a nexus between inhibitory or excitatory signals received from the periphery and the target regions of the CNS, and in addition, due to its connection between hypothalamus and paraventricular nucleus, also receives input from the vagus nerve. Thus, all meal-related metabolites, mechanical or chemical stimuli originated from the gastrointestinal tract are directly transmitted through the brainstem (Ritter 2004). Additionally, and like the median eminence of the hypothalamus, there is a highly vascularized area (the area postrema) mainly composed of fenestrated capillaries in which is located an area of binding sites for appetite regulatory peptides (Koda et al. 2005).

Biomarkers for the Study of Central Appetite Regulation in Humans

Appetite biomarkers are defined as physiological measures that relate to subjective appetite scores, food intake, or both (Horner et al. 2020). As a reference in this work, the proposed definition of a biological marker from the Biomarkers Definitions Working Group will be used, as follows: "*A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (Biomarkers Definitions Working Group 2001).

In addition, an appetite biomarker should be valid (clearly linked to appetite), reproducible, specific, sensitive, and feasible – measured in accessible or easily obtained material using ethical and minimally invasive methods (Horner et al. 2020).

The study of biomarkers of central appetite regulation has an obvious limitation, that is, access to sampling is limited, and only a few studies have evaluated the presence of biomarkers in cerebrospinal fluid (Tural and Iosifescu 2020). To solve this problem, various studies have used advanced imaging techniques, such as PET/CT or functional magnetic resonance imaging, so it has been possible to advance in the central regulation of appetite in humans (Smeets and Preissl 2016).

Therefore, it seems unlikely that it would be possible to find a gold-standard central appetite biomarker, given the complex multiple pathways involved in appetite regulation, both central and peripheral, acting at short or long-term, etc. In addition, the evaluation of appetite biomarkers is usually determined in people with complex and heterogeneous diseases, like obesity or eating disorders, which makes the use of these biomarkers even more difficult. Perhaps, the most appropriate approach is the combination of biochemical markers, such as the indicators of

nutritional status or neuroendocrine factors, together with functional neuroimaging tests and other complementary tests that will be discussed below.

Biochemical Biomarkers of Central Appetite Regulation

The usefulness of biochemical blood appetite markers has been demonstrated in numerous previous publications (Łucka and Wysokiński 2019; Tyszkiewicz-Nwafor et al. 2021). There is a huge diversity of factors involved in the regulation of appetite that circulate through the bloodstream, so it is sometimes advisable to study them according to how they act stimulating of inhibiting appetite.

Almost all appetite biomarkers are neurotransmitters. Among them, norepinephrine (NE) and endocannabinoids act to stimulate the sensation of hunger in the short term. NE is a neurotransmitter of the catecholamine family; it is synthesized by the neurons of the nucleus coreuleus of the brainstem and by the cells of the adrenal glands. The stimulating action of the sensation of appetite by this catecholamine is based on its action on the α2-adrenergic receptors of the hypothalamic paraventricular nucleus. NE action is based on stimulating the size of the meal more than the frequency of intake and induces a preference for foods rich in carbohydrates (Campese et al. 2017). Other molecules of hypothalamic origin that stimulate appetite at short term are endocannabinoids (anandamide and e-arachidonoyl glycerol). Its orexigenic action has been suggested based on studies using antagonists of its receptor, the so-called CB1, in which it has been observed that its blocking decreases intake, which suggests that they have a role in food intake (Rahman et al. 2021).

Opposite to the above biomarkers, there are parameters that stimulate the feeling of hunger at short term. These signals have their origin in the release of neurotransmitters that act centrally. The neuropeptide Y (NPY), the agouti-related protein (AgRP), the melanin concentrating hormone (MCH), the orexins A and B, (OXA and OXB), the galanin, and the central action of ghrelin stand out (Yoo et al. 2021).

NPY, a 36 amino acid neuropeptide, is considered one of the most potent orexigenic factors. It is mainly produced in the arcuate nucleus and its concentrations remain high in periods of fasting and progressively decrease during ingestion (Van Den Top et al. 2004; Shioda et al. 2008). Other molecule released by neurons in the arcuate nucleus is the AgRP. This molecule acts mainly at the level of the paraventricular nucleus and has great potential as an appetite inducer (Williams et al. 2001; Valassi et al. 2008). Its mechanism of action is based on the central blocking of the MC3 and MC4 receptors of the α-melanocyte stimulating hormone (α-MSH), a centrally secreted anorectic hormone (Cone 2005). An interesting characteristic of this protein resides in its flexibility regarding the duration of its action on the nervous system. Unlike NPY, which only has a short and intense period of action, the orexigenic stimulation of AgRP can act both short term and last up to 1 week after its release (Essner et al. 2017).

Prolonged fasting also stimulates the lateral hypothalamic area that produces melanin concentrating hormone (MCH), a 19 amino acid peptide that is also

Table 1 Summary of central biomarkers

	Orexigenic	Anorexigenic
Central biomarkers	NE GABA NPY AgRP Orexins A and B Galanin β-endorphines MCH Endocanabinoids Dynorphin	Serotonin CRF Dopamine Histamine Urocortin *POMC* *CART* *PrRP* *CRH* *Neurotensin* *α-MSH* *CGRP* *TRH* *PRP*

NE norpepinephrine, *GABA* gamma-aminobutyric acid, *NPY* neuropeptide Y, *AgRP* Agouti-related Peptide, *MCH* melanin concentrating hormone, *CRF* Corticotropin-releasing factor, *POMC* pro-opiomelanocortin, *CART* cocaine and amphetamine related transcript, *PrRP* prolactin releasing peptide, *MSH* Melanocyte-stimulating hormone, *CGRP* Calcitonin-gene related peptide, *TRH* Thyrotropin releasing hormone. Further information is described in the text

produced when low levels of leptin (an anorectic hormone involved in the suppression of appetite) are detected (Ramirez-Plascencia et al. 2017). Although multiple mechanisms of action of this peptide have been described at the central level, it has been shown that it has an inhibitory effect on the hypothalamic-pituitary-thyroid axis, thus reducing the energy balance of the individual (Kennedy et al. 2001).

The orexins (A and B) are neurotransmitters released by neurons in the perifornical area and the lateral hypothalamic area and are released in periods of fasting and hypoglycemia. These peptides stimulate appetite and glucagon secretion, inhibiting insulin secretion (Burt et al. 2011).

Galanin is a 29 amino acid neuropeptide that is in the CNS and the intestine. Although they have multiple functions, this neuropeptide has been described to be involved in the consumption of fats (Marcos and Coveñas 2021). These biomarkers are summarized in Table 1.

Image Methods to Study Central Appetite Regulation

Appetite is a behavioral multi-faceted sensation generated by a complex interaction of neural and hormonal signals, in which homeostatic, hedonic, and cognitive control pathways are involved (Berthoud et al. 2017). Thus, the physiologic "hunger" state interacts with some psychologic conditions (food preference, food intake behavior, the hedonic benefit, environmental and social context. . .) which at central level, induce a neural activity which may reflect different aspects of appetite. Thus, although the measure of these blood circulating neurotransmitters is quite interesting for the

evaluation of appetite, it is important to consider that these biomarkers do not show a real image of the neural activity of the central areas involved in appetite regulation.

Since early 1990, the use of neuroimaging techniques to functional studies about appetite and gut-brain interactions have become increasingly popular and at the present are an extremely valuable and promising research area, because they allow to establish a direct correlation between neural activity and appetite, and to study gut-brain interactions in live subjects (Loes et al. 1991; Lane et al. 1997). Nevertheless, the application of such techniques is not easy, because it also involves several practical and methodologic challenges which need to be arranged.

At the present, the most employed neuroimaging techniques which are employed to study central integration and regulation of appetite are the **Positron Emission Tomography** (PET), the **functional magnetic resonance imaging** (fMRI), **electroencephalography** (EEG), and **magnetoencephalography** (MEG).

Briefly in **PET**, a positron-emitting radioisotope is administered intravenously and distributed to tissues. Because the radioisotope readily crosses the blood brain barrier, it can be used to measure regional cerebral blood flow (rCBF). At the site of a brain activation, blood flow increases, which leads to greater uptake of the radioisotope into brain tissue, which results in an increase in the number of γ-rays detected at that site. Thus, with PET (and a related technique the single proton emission tomography (SPECT)), the local hemodynamic changes accompanying neuronal activity can be measured. As the use of PET alone has several technical limitations, it usually combines with a computed tomography (CT) which correct these drawbacks. In general, PET is considered as a quantitative method that depends on the kinetic of the radioisotope administered (Boecker and Drzezga 2016; Dodds 2017).

By the other hand, during a **fMRI** procedure, the subject is placed in a strong magnetic field, which magnetizes the tissues. Then, radiofrequency pulses are applied to excite protons (hydrogen atoms, chosen because they are abundant in biological tissues). On returning to a state of equilibrium, the protons emit radio waves, which are detected by a receiver coil. The time course of this relaxation process differs among tissues, and that difference is the source of contrast in MRI. In fMRI, the blood oxygen level dependent (BOLD) signal is used as a measure for neuronal activity. But although BOLD fMRI has better spatial resolution (1 mm), BOLD is a vascular signal and does not colocalize perfectly with spots of neuronal activation. As the BOLD fMRI cannot provide a measure of a single physiological parameter, a technical variation of this technique has been proposed to overcome this inconvenience, the arterial spin labelling (ASL) MRI (Alsop et al. 2015).

Pioneer neuroimaging studies were focused on the sensory properties of foods. Thus, the use of these techniques allowed to establish that aversive odors induced strong tracking signal in amygdalae and in the left orbitofrontal cortex (Zald and Pardo 1997). The use of more advance trackers were useful to study the tracker-intake distribution within the brain in either a low-carbohydrate or a moderate-carbohydrate high-protein diet (De Graaf et al. 2004; Lobley et al. 2021).

Regarding the study of central biomarkers of food intake, the studies using PET imaging showed a higher rCBF located in the prefrontal cortex in response to a meal (Lane et al. 1997), whereas several areas of the limbic or paralimbic areas (like

thalamus, hypothalamus, insular, and temporal cortex), caudate nucleus, and cerebellum the rCBF was lower. These results suggest prefrontal cortex may be involved in the onset of satiety whereas the relationship of the rest of areas in the hunger and satiety sensations are still under investigation (Haase et al. 2011; Sun et al. 2015).

About the use of MRI, several studies have shown a high reduction of BOLD fMRI signal in the hypothalamus in response to an oral glucose ingestion (Matsuda et al. 1999; Smeets et al. 2005), others did not (Purnell et al. 2011). Numerous studies have also tried to determine the cerebral regions activated during the fasted and fed states. They found that, in response to a visual or tactile stimulus (food vs non-food or high calorie vs low calorie), there was an increase of the activity of the amygdala, orbitofrontal cortex and the insula (Goldstone et al. 2009; Menon and Uddin 2010) and a decrease in activity as BMI increases (St-Onge et al. 2005). Overall, these results suggest that in response to orexigenic signals (fasted status) there is an increase of BOLD activity in several brain regions whereas the anorexigenic signals (fed status) induce a decrease in the same areas.

The recent studies that used PET and fMRI techniques to study brain activity clearly showed neural correlates between the pleasantness of foods and changes in rated pleasantness of foods during meal consumption. Nevertheless, there are limitations on the use of these techniques. Thus, fMRI and PET scans and analyses are not easily to carried out or are not widely available and are relatively expensive. Data from fMRI and PET scans as indirect indicators of neural activity, cannot be considered as causal factors which lead it to satiation. Additionally, these techniques can only be performed in subjects in the supine position with restricted movement. Therefore, under these circumstances, the measurements and the results are rather artificial. For these reasons, it is unlikely that these techniques will be routinely used to support a claim for the satiety-enhancing capability of functional foods, but undoubtedly they represent an exciting contribution to understanding the biology of food choice and intake regulation (Blundell et al. 2010).

Another interesting focus to study central regulation of appetite is by measuring the electrical activity of the neurons in the brain. The **EEG** measures electrical activity through electrodes placed on the head, whereas **MEG** measures the magnetic signals which correlate with electrical signals from neurons by using a helmet with superconductive sensors on the head (Smeets and Preissl 2016). The advantage of these techniques is that they can operate at a millisecond scale, so they can measure direct interactions between different areas of the brain without delays, but their main disadvantage is the difficulty to detect neuronal activity from deep areas of the brain (like hypothalamus).

Regarding EEG, the first approach was described in 1980 (Marcos and Coveñas 2021). As several reports described potential approaches with measuring EEG activity with presentation of images. Thus, several reports define the optimal EEG wave parameters to study the visual perception of food by subjects (Pergola et al. 2017), whereas others measured evoked gamma oscillation (30–80 Hz) to study perception of food at gustatory and visual modality (Domracheva and Kulikova 2020). By the other hand, recent studies which employed MEG suggest that the appetite behavior may be driven mainly by unconscious decision-making processes

(Forman et al. 2018), and the presentation of visual food signals beware the threshold of awareness may reflect appetite behaviors (Takada et al. 2018).

Behavioral Measures of Central Appetite Regulation

It should be noted that biomarkers are unable to fully characterize the range of processes involved in appetite control and should only be used to make claims about appetite in combination with behavioral measures. It has been clearly established that the regulation of appetite is carried out not only by neuroendocrine factors, but that other psychological, educational and even religious factors intervene in the decision to eat food or not. In addition, the concept of appetite is subjective, so it is somewhat logical to use biomarkers that also subjectively evaluate these appetite sensations (Ziauddeen and Fletcher 2013).

These aspects are of great importance, especially in reference to the development of drugs with potential appetite-suppressing action. Unfortunately, experience has shown that since appetite regulation is mainly carried out at the central level, drugs that act in this way, although effective, have a variety of side effects that limit, or even better, avoid their use in humans, as has been the case with rimonabant or sibutramine (Cheung et al. 2013; Sloan et al. 2017). In these cases, the evaluation of these subjective biomarkers of appetite may be of great interest.

The most frequent employed method is the use of appetite ratings through visual analogic scales (VAS) (Stubbs et al. 2000). This method allows monitoring the subjective hunger/satiety feelings in laboratory and free-living conditions, although the former may be of greater interest. The use of VAS in laboratory assays are usually performed with test meals or with ad libitum consumption of different meals (Thivel et al. 2018). Ad libitum meals, also known as buffet meals, can additionally be used to evaluate effects on specific food categories and food preferences. Preliminary studies were carried out with the traditional pen and paper technique, although the current development of electronic devices has allowed the development of applications for smartphones, tablets, etc., which greatly facilitates data collection and reliability (Hernández-Morante et al. 2016) (Fig. 3).

An interesting test to evaluate behavior measures of appetite is based on eye-tracking. In the work of Frayn et al., participants' eye fixations were tracked and recorded throughout 8-s presentations of displays with healthy food, unhealthy food, and non-food images before and after a sad mood induction (Frayn et al. 2016). Interestingly, the authors concluded that sad mood made subjects less fixated on healthy foods (Frayn et al. 2016). Within a similar procedure, Motoki et al. showed that the induction of anxiety, but not anger or neutral feelings, led to greater visual attention on hedonic foods only (Motoki et al. 2019).

Other interesting test to examine subjective appetite behavior is based on attention bias measures. In this test, a food-related and a non-food related pictures are shown in a screen for a short period, and then an image is replaced by a dot probe. The participants must press the right key corresponding to that position. When the reaction time is longer in food images than in non-food related images, it is assumed

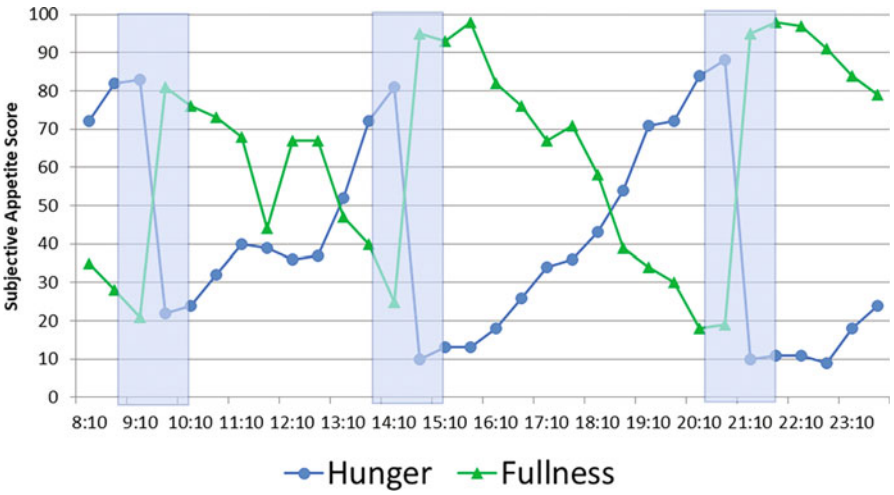

Fig. 3 *Example of the evaluation of appetite ratings of a volunteer on free-living conditions.* Subjective scores in a 0–100 scale. Blue line shows subjective "hunger" and green line subjective "satiety." Blue boxes represent mealtime. A shift between hunger and satiety can be observed in those mealtimes

that there is an attention bias toward food. Through this test, it has been possible to demonstrate the effect of several drugs for the treatment of obesity (still under development) to reduce this attention bias toward food (Chamberlain et al. 2012).

There are other tools of interest, but among them it is worth highlighting the Leeds Food Preference Questionnaire (LFPQ) (Finlayson et al. 2011). This tool is used to evaluate different aspects related to the reward potential of food. This test consists of two parts: an explicit measure of taste and desire for foods from different categories (sweet, salty, high-fat, or low-fat), and an implicit measure of food cravings through a test in which they are shown by pairs pictures of foods that belong to different groups. The participant must choose which of the two food images she would like to eat at that moment. A greater reaction to unhealthy foods (for example, high-fat sweets) indicates an implicit high desire for those foods (Griffioen-Roose et al. 2011). This test has been employed in energy-depletion studies (Thivel et al. 2018), fasted and post-prandial states (Ruddick-Collins et al. 2019), as well as in the study of the influence of exercise on appetite (Fillon et al. 2020).

Biomarkers for the Study of Peripheral Appetite Regulation in Humans

Peripheral Signals that Regulate Appetite

Regarding peripheral biomarkers of appetite regulation, one interesting aspect which may be considered is the effect of physical events which are related with appetite regulation. In this sense, the weight or volume of food has been shown to be related

to the size of meal size and may be considered as a biomarker of satiation (Poppitt and Prentice 1996). By the other hand, the progressive filling of the stomach and intestine induces mechanical (dilation) and chemical signals (induced by the concentration of nutrients) that are detected by mechano- and chemoreceptors of the digestive tract, which send a signal that is transmitted through the vagus nerve, to the nucleus of the solitary and ventromedial tract, which in turn generates a satiety response (Wu et al. 2020). Additionally, the progressive filling of stomach and guts stimulate the secretion of numerous hormones (CCK, GLP-1, ghrelin, PP), which have a direct effect on gastric depletion or develop a long-time postprandial effect of satiety (Koliaki et al. 2020). Additionally, it has been observed that a decrease in oxidation or body temperature is also associated with hunger. Thus, thermogenesis may reflect the level of energy consumption (oxidative phosphorylation and ATP consumption in the liver) by the subject (Cronise et al. 2017) and may serve as a potential biomarker of satiety (Table 2).

The composition of food may also affect the intensity and duration of hunger and satiety. Proteins are the macronutrients with the greatest satiating power, either by inducing the secretion of cholecystokinin (CCK) or by direct action on the CNS or hepatic or portal receptors (Veldhorst et al. 2008). Carbohydrates have a similar satiating capacity, stimulating the secretion of peripheral molecular signals (GLP-1 or amylin) and delaying the transit of the digestive tract (Salvatore et al. 2019). Fats, on the other hand, are the macronutrient with the least satiating power. Not only the composition of food, but also changes in blood glucose, physiological stores of fatty metabolites or amino acids can be recognizable signals by hypothalamic neurons that would mark the start or end of the meal (De Graaf et al. 2004).

Table 2 Summary of peripheral biomarkers

	Orexigenic	Anorexigenic
Peripheral biomarkers	Ghrelin Thyroid hormone Glucocorticoids Apelin	CCK Enterostatin GLP-1 Somatostatin Amylina GRP PYY Leptina Insulin Motilin Interleukins PRP Motilin Galanin-like peptide PTT Obestatin Nesfatin-1 Amylin PP

CCK Cholecystokinin, *GLP* Glucagon-like peptide, *GRP* Gastrin releasing peptide, *PYY* Peptide YY, *PTT* Peptide Tyrosin-Tyrosin, *PP* Pancreatic polypeptide

Regarding hormonal biomarkers of appetite regulation, several components have been proposed:

Ghrelin is a polypeptide mainly secreted in the gastric fundus. Ghrelin targets the hypothalamus, specifically the arcuate nucleus, where it stimulates different neuronal subpopulations that release other regulatory peptides (neuropeptide Y (NPY) and agouti-related protein (AgRP)). In addition to regulating appetite, ghrelin has been found to be involved in learning, in glucose metabolism, and is involved in stimulating gastrointestinal motility and gastric secretion. Although it is evident that its administration in humans induces hunger, it is considered a molecule that acts as a link between the short- and long-term mechanisms of ingestion due to its competitive interaction with leptin and is also an endogenous agonist of somatotropin (Beck and Pourié 2013; Lv et al. 2018).

Triiodothyronine (T3) and **thyroxine** (T4) are hormones secreted by the thyroid, which have the regulatory function of basal metabolism, reducing fat reserves and lowering leptin and insulin levels (Mullur et al. 2014). Lastly, endogenous glucocorticoids have an antagonistic effect to that of leptin and insulin, which is why they also have a central hunger-stimulating action (Adam and Epel 2007).

By the other hand, there also are peptides that are mainly synthesized in the digestive tract, that act at the central level, specifically at the level of the nucleus of the solitary tract, either through peripheral innervation (vagus nerve) or released into the circulation through an endocrine mechanism. The most relevant peptides are cholecystokinin (CCK), enterostatin, glucagon-like peptide (GLP-1), amylin, gastrin-releasing peptide (GRP), and pancreatic polypeptide (PP) (Parker et al. 2014).

CCK is a 33 amino acid protein that is synthesized peripherally from the duodenum in the presence of fats and carbohydrates and centrally from the hypothalamus. This protein exerts its anorectic action through the activation of its specific receptor (CCK-A), inducing a pyloric contraction that causes a delay in gastric emptying, which causes a vagal stimulation that reaches the nucleus of the solitary tract, inducing satiety (Wang et al. 2000; Wu et al. 2020).

Enterostatin is a 5 amino acid oligopeptide that is produced in the intestinal lumen by the action of trypsin on its precursor (procolipase) and is involved in the selective inhibition of fat intake (Berger et al. 2004).

GLP-1 is released by the action of the enzyme proconvertase on the precursor synthesized by the "L" cells of the ileum during the ingestion of foods, especially rich in carbohydrates. At the peripheral level, it inhibits gastric emptying and glucagon secretion, stimulating insulin secretion, and at the central level it acts on the paraventricular nucleus, stimulating satiety (Andersen et al. 2018; Goyal et al. 2019).

Amylin is a peptide secreted by the ß cells of the pancreas, with a peripheral action mechanism complementary to insulin and centrally stimulating satiety (Trevaskis et al. 2013).

On the other hand, **GRP** is synthesized by endocrine cells of the gastric mucosa, stimulates the release of gastrin at the peripheral level and delays emptying, while it inhibits appetite at the central level (Rocca and Brubaker 1999).

Finally, **PP** is a peptide released by the PP cells of the islets of Langerhans by postprandial vagal stimulation, and exerts its anorectic effect at the central level, apparently independent of gastric motility (Holzer et al. 2012).

The above factors exert their action at short-term. In addition, there is also a long-term regulation of appetite performed by peripheral factors. The two main factors involved in the long-term regulation of appetite are leptin and insulin, considered the main "adiposity signals" in the body (Suzuki et al. 2012). In fact, changes in the plasma levels of both hormones indicate alterations in the body's energy state and its level of fat reserves, which makes the CNS respond by regulating appetite/intake to restore the levels.

Leptin is a 146 amino acids peptide that is produced in adipose tissue and in the stomach. This hormone is present in circulating blood in proportion to the level of body fat (the greater the amount of fat stored, the higher the levels of circulating leptin), and its central function is the inhibition of appetite and regulation of body weight (Ma et al. 2004). It has the particularity that its secretion follows a circadian rhythm, with a maximum level of secretion in diurnal animals during the night and minimum during the day, and they are usually higher in women than in men (Ahmad et al. 2001). Leptin reaches levels in the cerebrospinal fluid similar to those present in the blood, reaching to the hypothalamus and stimulating the arcuate, ventromedial, dorsomedial, and paraventricular nuclei, stimulating the inhibition of orexigenic neuropeptides (NPY and AgRP) and stimulating anorectic molecules (pro-opiomelanocortins (POMC) and transcript regulated by cocaine and amphetamines (CART) a central level). In addition, it has been observed to regulate energy expenditure and lipolysis (Berthoud 2012).

Insulin is a 51 aa hormone synthesized by pancreatic beta cells, it is considered a very important element in the regulation of glucogenic and energy homeostasis. Similar to leptin, its blood levels depend on the blood levels of glucose, amino acids, and its antagonistic hormone (glucagon), in addition to being released in stages of stress. In the CNS, it acts at the level of the arcuate and ventromedial nuclei, reducing the sensation of appetite, stimulating the activity of anorectic signals (CCK and CRH), inhibiting gluconeogenesis and stimulating lipolysis (Seoane-Collazo et al. 2015). Paradoxically, the insulin action at the peripheral level decreases blood glucose levels, stimulating appetite (Strack et al. 1995), which, like leptin, constitutes a link between appetite regulation and metabolic regulation.

Peptide YY (PYY) circulates in two forms: 1–36 (predominant in periods of fasting) and 3–36 (released during ingestion). The most important is form 3–36. It is a gastrointestinal hormone secreted by the L cells of the intestine that belongs to the NPY family, exerting an antagonistic action to it, acting at the arcuate nucleus of the hypothalamus, inhibiting the sensation of appetite and the level of intake (Batterham et al. 2002).

Corticotropin Releasing Hormone (CRH) is a 41 amino acid neuropeptide, produced mainly in the paraventricular nucleus and also regulated by leptin levels, it reduces NPY expression and consequently intake (Gioldasi et al. 2019).

Metabolomics and Central Appetite Regulation

In addition to all the factors that have been discussed above, the new 'omics' techniques should provide information of great interest regarding the regulation of appetite, and therefore could be used as biomarkers. From all of them, metabolomics is the one that has attracted most attention regarding the study of appetite, since most of the small molecules or metabolites present in biological samples derive from metabolism associated with food intake (Collins et al. 2019) (This topic has been deeply reviewed in the work of Horner et al. (2020)).

Several nutrient-related metabolites, such as amino acids, lipids or carbohydrates, have been proposed as key molecules associated with appetite control for more than 60 years, and are among the main biomarkers of appetite (Table 3).

Fatty Acids and Their Derivatives as Biomarkers of Appetite Regulation

The role of free fatty acids (FFA) or non-esterified fatty acids (NEFA) on appetite regulation has been established from long-time ago (Karhunen et al. 1997). Regarding NEFAs, although there are studies which suggest a role of them in the duration of satiety (Gatta et al. 2009), other studies establish such relationship only under certain conditions (Deighton et al. 2019). By the other hand, although multiple experimental models suggest a role of FFA in appetite control, their role in human nutrition is still not clearly determined (Walker and Remley 1970; Little et al. 2007). Similarly to

Table 3 Potential metabolites used as biomarkers of appetite regulation

Family	Metabolites
Lipids and derivatives	Free Fatty Acids Non-esterified fatty acids Short-chain fatty acids Oleoylethanolamide Anandamide Palmitoylethanolamide Cholesterol Cholesterol-derived hormones (cortisol, etc.)
Amino acids and derivatives	Total serum amino acids Histidine Glutamate Valine Isoleucine Leucine Tryptophan Aminobutiric acid Taurine Creatinine
Carbohydrates and derivatives	Fasting plasma glucose Fructose
Other metabolites	Ketone bodies Lactate Bile acids

FFA, the few studies performed with short chain fatty acids (acetate, butyrate or propionate) pointed to a possible relationship with appetite regulation, although the evidence is still unclear (Byrne et al. 2015).

Within the fatty acids derivatives, ethanolamides, a class of lipid signaling molecules derived from fatty acid precursors, such as oleoylethanolamide (OEA), anandamide (AEA), and palmitoylethanolamide (PEA) have attracted more attention. Thus, OEA seems to be positively associated with satiety and fullness (Kong et al. 2016) and AEA and PEA seem to have some relationship with postprandial hunger state (Rigamonti et al. 2015), but the knowledge about the exact mechanisms is still limited.

Amino Acids as Biomarkers of Appetite Regulation

The relationship between the concentration of circulating amino acids, peptides, protein, and appetite control have been long studied. Thus, as far as in 1956, Melinkoff et al. described a reciprocal relationship between the serum amino acid concentration and appetite (Mellinkoff 1997). Nevertheless, it seems that this association is evident only under certain conditions (like a concomitant action between amino acids and gut peptides like CCK or GLP-1) released during satiety. Thus, there should be complementary pathways involved in such regulation (Neacsu et al. 2014).

By the other hand, although there are studies which establish a relation between postprandial levels of all 20 amino acids and suppression of food intake (Luscombe-Marsh et al. 2016), other studies highlighted the potential role of individual amino acids (like histidine, valine, isoleucine, leucine, and tryptophan) in food intake (Okusha et al. 2017; Solon-Biet et al. 2019). Overall, even if circulating amino acids could be considered as potential biomarkers, this might be true under certain contexts directly or indirectly (like an effect mediated by gut peptides) (Horner et al. 2020).

Glucose and Other Carbohydrates as Biomarkers of Appetite Regulation

The role of glucose in appetite control have been extensively investigated (Smith and Campfield 1993; Campfield and Smith 2003), and there is still controversy about its specific role. Thus, whereas there are many reports which suggest a role of glucose levels and appetite rating (Melanson et al. 1999), others did not (Schultes et al. 2016). This may be explained on basis of glucose may exert its regulatory effect in those cases of extreme situations (like hypoglycemia or hyperglycemia), but not within physiological rates (Horner et al. 2020). Thus, it is still under research the value of glucose as a potential biomarker of appetite control.

Other Metabolites Proposed as Biomarkers of Appetite Regulation

Ketone bodies (like circulating β-hydroxybutyrate) have showed a strong inverse correlation with the reduction of orexigenic signaling and positive correlation of fullness, although the exact mechanism (probably by effect on gut hormones) is still under investigation (Sumithran et al. 2013). Regarding lactate, the current evidence suggest that may have a role in appetite regulation under determine conditions and

seems to depend on the circulating levels of other metabolites (such glucose) (Lam et al. 2008) through a mechanisms which may involve ghrelin secretion (Engelstoft et al. 2013). Lastly, although there are studies which propose the use of bile acids as potential biomarkers of appetite regulation, the current findings in humans are very limited (Kuhre et al. 2018).

Conclusion and Future Directions

As it has been tried to be commented above, appetite regulation is performed through a complex interaction of external and internal factors to regulate the drive to eat. Within these internal factors, those generated at the central and peripheral level can be differentiated. In this context, it is very difficult to recommend a biomarker for the study of appetite, and it would be advisable to combine subjective measures, neuroimaging measures, and other biochemical parameters. Nevertheless, the study of appetite biomarkers is continuously growing, and new parameters are constantly suggested as suitable indicators to measure appetite. The introduction of omics techniques, especially metabolomics, will undoubtedly provide more information. It is expected that soon there will be adequate indicators to confirm the ability of new pharmacological and behavioral therapies, etc., to manipulate the appetite and thus reducing the incidence of diseases such as obesity.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, the main peripheral and central biomarkers of appetite regulation have been reviewed. These biomarkers are central to the development of novel interventions, both pharmacological and behavioral, for obesity treatment. In addition, multifactorial regulation of appetite is highlighted, including external as well as internal factors. Therefore, the need to accurately evaluate all these factors to determine the appetite sensations is discussed. Considering that appetite is a subjective perception, the use of accurate tools as those described and neuroimaging techniques is mandatory. In addition, novel omics techniques may bring some new information to increase the knowledge in this area. Preceding available drugs for obesity treatment targeting appetite have failed due to their adverse effects. The development of new antiobesity drugs should consider these external and subjective factors before becoming available for clinical use.

Mini-Dictionary of Terms

- **Appetite.** Appetite is a subjective sensation, a desire that prompts the individual to satisfy the desire to eat.

- **Central Appetite Regulation.** Neurohormonal signals, like neuropeptide Y, agouti-related protein, etc., produced or acting in the central nervous system involved in appetite regulation.
- **Hypothalamus.** A small area located below the thalamus, in the center of the brain, and is considered the regulating center for hunger and satiety. It is structured in several nuclei: the arcuate, paraventricular, ventromedial, and dorsomedial nuclei and the lateral hypothalamic area
- **Metabolomic.** Comprehensive analysis of different metabolites in a biological specimen. Regarding appetite, this technique allows the determination of multiple nutrients simultaneously.
- **Neuroimaging techniques.** High resolution diagnostic imaging techniques, mainly PET/CT and fMRI, which are employed to evaluate brain activity.
- **Overweight.** Situation characterized by a body weight higher than what is considered as healthy. Body mass index is usually employed to diagnose the presence of overweight.
- **Obesity.** A disease characterized by an excessive body fat accumulation that increase the risk of morbidities and mortality. This disease is the result of an inadequate appetite regulation, since it is a consequence of an inability to balance energy intake with energy expenditure.
- **Peripheral Appetite Regulation.** Hormonal, biochemical, and physical factors, produced by the peripheral organs (gut, stomach, etc.) involved in appetite regulation.

Key Facts of Appetite Regulation

- An inadequate appetite regulation underlies various diseases, especially overweight and obesity.
- Despite a huge number of neurohormonal factors, in humans, external factors like social, psychological, and even religious factors may determine food intake
- Appetite is regulated by both peripheral factors, secreted by the stomach, gut, pancreas, etc., as well as by central factors secreted by specific brain areas.
- Since appetite is a subjective representation of a physiological need, it is impossible to evaluate appetite only with biochemical parameters, and other tools like neuroimaging techniques and behavioral measurements are of interest.

Summary Points

- *Appetite is a subjective sensation that prompts an individual to satisfy the desire to eat*
- *Appetite is regulated by internal (neurohormonal factors) and external (social, educational, psychological, religious,* etc.*) signals*
- *Internal signals can be divided in central factors, like neuropeptide Y or POMC, and peripheral factors like ghrelin and leptin.*

- *Considering the subjectivity of appetite, the use of complementary techniques like imaging or behavioral measurements is mandatory.*
- *Metabolomic and other omic techniques should provide the necessary information to better understand the regulation of appetite.*

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Growth Reference Charts as Biological Indicators of Nutrition

33

Head Circumference

Muhammad Aslam and Muhammad Asif

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Abstract

Growth monitoring is a useful measure which can be broadly contributed to the promotion of child health and nutrition. It is also used to diagnose chronic systematic and endocrine disease at any early stage. Human growth is age-related and occurs at different rates over different time periods. From birth to adolescent, it covers at least following four distinct phases, i.e., infancy, childhood, juvenile, and

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adolescent. Human growth is said to be normal when progression changes in growth variables are well-matched with the established standards for a given/reference population. For this reason, growth reference charts are very useful tool to study a country's health profile and biological indicators of nutritional status.

Different anthropometric measurements are used to reflect growth of a child and its related issues. However, the measurement of head circumference has a crucial role in the pediatric growth and clinical examination for a child. It is used to evaluate the nutritional status and a proxy measure for brain development of a child. Thus, construction of growth reference charts for the head circumference is very useful tool for the practicians and other health practitioners. Usually, three different ways, i.e., Z-scores, percentiles, and percent of median, are used to express the frequency distribution of the measurements in the growth charts. The popular methods to construct these charts are the LMS method and quantile regression.

Keywords

Biological indicator · Body mass index · Growth reference charts · Head circumference · LMS method · Neck circumference · Nutritional status · Quantile regression

Abbreviations

BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CI	Conicity index
HBCS	Helsinki Birth Cohort Study
HC	Head circumference-for-age
HRY	Healy-Rasbash-Yang
LMICs	Low-income and middle-income countries
LMS	Lambda-Mu-Sigma
MAM	Moderate acute malnutrition
MUAC	Mid-upper arm circumference
NC	Neck circumference
NHANES	National Health and Nutrition Examination Survey
QR	Quantile regression
SAM	Severe acute malnutrition
SD	Standard deviation
WC	Waist circumference
WHtR	Waist-to-height ratio
WRC	Wrist circumference

Introduction

The health profile of any country can be reflected most accurately by somatic growth of its children. A numerical increase in size or mass of a body dimension is referred to as growth in humans, and human growth is an important and delicate predictor of

individual's health and nutrition (Bogin 1999). It is a complex biological process that is regulated by genes and endocrines (de Onis et al. 2006; Asworth et al. 2008). Human growth is said to be normal when progression changes in growth variables are well-matched with the established standards for a given population. Human growth is age-related and occurs at different rates over different time periods. From birth to adolescent, it covers at least following four distinct phases, i.e., infancy (birth to 2 years of age), childhood (2 to 7 years of age), juvenile (7 to 12 years for boys and 7 to 10 years age for girls), and adolescent (at the end of previous stage up to 18 years of age) (Asif 2018).

Growth during infancy and childhood age, in all its aspects, is the priority concern to all (Eveleth and Tanner 1990). A well-growing child is most expected to have healthy immunological defense against any infections. Furthermore, better growth of a child may also decrease the risks of child mortality, case fatality rates, and some severe infections. Such a healthy growth in result will increase the human capital and produce a youth with a greater potential for becoming a productive member of a society. Physical growth and cognitive development during fetal life, infancy, and childhood age are directly influenced by some genetic, hormonal, nutritional (i.e., ill nutrition by maternal and child), and environmental factors, e.g., ethnicity, gender difference, socioeconomic status, seasons, and parity (Towne et al. 2006; Schell and Knutson 2006).

The study of the growth monitoring is to be essential to get the information about health, either individually or collectively. Growth monitoring is a useful measure which can be broadly contributed to the promotion of child health and nutrition. It is also used to diagnose chronic systematic and endocrine disease at any early stage. It has the potential for significant impact on mortality even in the absence of nutrition supplementation or education (Garner et al. 2000). Different countries use growth monitoring with the same objective as preventing growth retardation with timely and early detection of faltering growth (Morley 1973). Over the last few decades, growth monitoring has been used in different ways which are of the following:

- For the assessment of nutritional status of individual children or different populations. It also makes some health comparison between populations.
- For detecting undernourished children who should be included in supplementary feeding programs.
- For identifying children with health problems that cause growth faltering and might need some treatment.
- For providing some guidance to parents about their child nutrition and to motivate them about the improvement of healthy childcare practices.

In the low-income (underdeveloped) countries, the primary focus of growth monitoring is the identification of malnutrition while the developed countries mostly aim to retrieve growth disorders such as growth hormone deficiency, turner syndrome, and celiac disease (van Buuren et al. 2004). Nutritionists and health program planners in the developing nations consider that growth monitoring is not living up to mark for contributing to child survival and development. Because in these nations,

a considerable segment of the total population resides in rural areas where people do not have access to proper health facilities. Therefore, for employment and other better opportunities, people generally use to migrate to urban settlements. In these circumstances, monitoring of individual children becomes inaccurate and measurements with regular intervals are also impossible. It is also difficult to judge whether nutritional status of children in this particular community has improved over a period of years (Cole 1997).

Proper growth monitoring consists of the regular measurements of growth-related variables, i.e., height and weight over time so the growth velocity can be assessed. An adult health is totally a reflection of his/her health as a child. If the height of a child is not increasing as it should, then the child's weight loss may have an underlying medical problem or a genetic defect (Tanner 1986). To study the growth pattern of any population, we find the reference values of different anthropometric variables and make their charts, namely called the growth charts. Different experts have developed the growth standards for children, their interpretation and have provided some guidelines to use them (Waterlow et al. 1977). Prior to discussing about the growth reference values and growth charts, we describe the anthropometry and the importance of anthropometric variables for the assessment of growth and nutritional status of a child.

Biological Indicators of Nutrition

Anthropometry is the most practical tool used worldwide for assessing the growth or nutritional status of an individual or population. It is mainly concerned with the measurement of physical sizes and shapes of human body and is used for understanding the human biological variability over time (WHO 1995). Different anthropometric measurements have their own use and importance. Many locally or nationwide nutritional surveys normally contain height and weight measurements with other attributes such as age and sex. For infant's and child's growth monitoring, three most extensive and internationally recommended anthropometric indicators are employed viz. weight-for-height (wasting or obesity) that measures body weight relative to height used as current nutritional status, height-for-age (stunting) reflects cumulative linear growth used as a past or long-term inadequacies nutrition. These both indicators are also called primary indicators of nutritional status. Whereas the third indicator, weight-for-age (under or overweight) reflects body mass relative to age has been widely used to assess the changes in the magnitude of malnutrition over time (WHO 1995, 2007; Alderman 2000). Head circumference (HC)-for-age and mid-upper arm circumference (MUAC)-for-age are also two other important indicators used for determining the nutritional status of the children under 5 years of age (Asif 2018; Asif et al. 2017, 2018a, b).

Evaluation of nutritional status during childhood and the juvenile years is very important and necessary. Because in these years, a remarkable variation occurs in the level of hydration and the lean and adipose tissues may increase the risks of getting obesity or other metabolic risks (Talwar et al. 2010; Wells et al. 1999). For this

purpose, many researchers in globe also recommend body composition analysis to diagnose obesity (Rebato 2003). They suggest different anthropometric parameters for obesity. Body mass index (BMI) has long been reputed criterion used for defining general obesity in both children and adults (WHO 2000). To know about the detrimental effect of regional body fat, i.e., abdominal and intra-abdominal fat, the values of waist circumference (WC), waist-to-hip ratio (WHR), waist-to-height ratio (WHtR), and conicity index (CI) are used as indicators of central obesity (Valdez et al. 1993; Mokha et al. 2010. Physicians often use all of these measurements in clinics because numerous risks, particularly hypertension, diabetes mellitus, dyslipidemia, coronary heart diseases, and cancers are connected with obesity (Marinou et al. 2010). However, all these measurements have some practical barriers in clinical measurements; clothing is one major disturbing factor complicating during measurements (Wills and Bhopal 2010).

To overcome these obstacles, some newly added anthropometric parameters, i.e., neck circumference (NC), wrist circumference (WRC), and MUAC, have been proposed as reliable indicators of upper-body obesity and insulin resistance as well as cardiometabolic risk in children and adults (see, for example, Ben-Noun and Laor 2006; Tatar et al. 2014; de Almeida et al. 2003, and many others).

In addition to these parameters, the HC (cm) has a crucial role in the pediatric growth and clinical examination. The HC is used for growth monitoring of the brain because cognitive functions, intra-cranial volume, and brain volume are closely related to the magnitude of HC (Bartholomeusz et al. 2002; Bray et al. 1969). A rapid increase in the HC is usually observed within the first few years of life which marks histological changes in the brain. Thus, it is suggested that the HC measurement in children should be performed with care and should be recorded regularly (for more details, see Aslam et al. 2019 and the references cited in).

After reviewing the importance of different anthropometric parameters, we conclude that all these biological indicators deserve serious attention of the researchers in terms of the assessment of growth and nutritional status as well as the risks of many diseases.

Growth Reference Charts

In epidemiological field, growth chart is considered as an important tool that represents the growth pattern of different anthropometric measurements of a child in the form of reference curves. Reference curves, drawn on a chart, represent the distribution of reference measurements at each age. For proper child's growth assessment, an appropriate growth reference or standard is required. A reference or a standard is a group of values (centiles) that defines the statistical distribution of anthropometric measurements for some population. These values are plotted against age on a chart. Deviances from reference values may exhibit some problems in growth and need some further investigations (Cole 1990, 2007). The first growth references were established in England in 1906 (Tanner 1981).

An inadequate reference may also lead the researchers to false conclusions for a child or population and even to wrong treatment or interventions. Some secular changes in growth (i.e., changes in average size and body mass) also motivate the researchers that growth references should be updated on regular basis. Cole (2006) also reported that growth references must be updated after 15 years.

Growth standards and growth references are two different things. Firstly, a growth reference has a descriptive approach while a growth standard has prescriptive approach. Secondly, growth reference describes how children of a certain population grow in a certain time and it is not universally applicable to the other populations. Whereas growth standard describes how children should grow and these standards are made by choosing the sample of children who are well nourished and who grow in optimal environmental conditions. These references are universally applicable to the other populations (Garza 2006).

In the developed countries, many large nationwide studies related to growth of children have been undertaken. For instance, Hong Kong firstly designed growth charts for its children in 1960s and then updated in 1985 and 1993. Similarly, the growth curves for the UK children and US children were developed in 1980s and then were further revised in 1990 and 2000, respectively (Eveleth and Tanner 1990; Freeman et al. 1991; Kuczmarski et al. 2002). Over a longer period of time, these growth curves for the US and UK children have been used as reference standards in several countries. On the other hand, a long-running debate among auxologists, public health workers, and nutritionists also has been about the desirability of growth reference for each nation, separately or otherwise of a single universal reference or standard for growth. Finally, they decided that each country should construct their own reference values or standards for growth because of the larger differences between populations. In addition, there is no guarantee that all the populations have the same growth potential (Eveleth and Tanner 1990).

It is clear that due to genetic disparity in origin or environmental differences, the American or British standard shall not truly reflect the growth of the Japanese or any other country (Goldstein and Tanner 1980; Baldwin 1988). Children from the developed Western countries, on average, are taller and heavier than the Asians. The mean birth weight of singleton term Pakistani, Indian, and Bangladeshi infants are less than the mean birth weight of individuals participated in the UK Millennium Cohort study (Yajnik et al. 2003; Janjua et al. 2009).

Few studies, comparing weights and heights of children with different ethnicities, report that the African-Caribbean children are taller and heavier than the Caucasian children. While the Asian and Chinese children are shorter and low weight than the Caucasians. Comparing to the English general population, mean BMI among Afro-Caribbean girls and boys are higher, while the Chinese, Indian, and Bangladeshi boys and girls have lower mean BMI. The ethnic disparity has showed that growth centiles of developed countries may not be used for clinical purposes in developing countries which may lead to false conclusions on child or population well-being and even to wrong treatments or interventions (Kelly et al. 2009; Stanner 2001).

After that, a number of studies have been undertaken in different developed countries including Italy (Cacciari et al. 2002), Brazil (Silva et al. 2010), and Sweden

(Wikland et al. 2002) for reporting their own growth reference values of different nutritional parameters. The Asian developing countries like Iran (Ayatollahi and Carpenter 1991), India (Sharma et al. 2007), and Pakistan (Mushtaq et al. 2011; Asif et al. 2018b, 2020; Aslam et al. 2019) have also designed nationwide studies to develop their own growth reference values for determining the local growth pattern and to identify undernourished communities.

Types of Growth Reference Charts

The position of a child's growth measurement is related to the population to which he/she belongs, and the trend of his/her growth performance can be visualized through a growth chart. Three different ways, i.e., Z-scores (or standard deviation (SD) scores), percentiles, and percent of median, can be used to express the frequency distribution of different anthropometric indices in the growth charts.

Z-score (SD Score)

It expresses the measurement's position in a reference population as a standard deviation from the median of a reference population. The main advantage of using the Z-score is that mean and standard deviation can be calculated for groups of Z-scores and interpreted under normality (WHO 1995; Pere 2000).

Percentiles

Percentiles or simply centiles depict that how many percentages of reference children are above the individual children's measurement, e.g., for height measurement, 50th centile shows that 50% of children are shorter and 50% children taller than the 50th centile. Centiles are generally easy to understand, and their interpretation is also easy, but their means and standard deviations cannot be calculated. Moreover, centiles do not accurately classify children who are at extremes of the distribution (Waterlow et al. 1977; WHO 1995).

Percent of Median

Percent of median expresses how many percentages the individual's measurement is from the reference population's median. It is also easy to understand but it lacks exact correspondence with a fixed point of the distribution across ages and cut-offs for different indices are not the same (WHO 1995).

For growth assessment, seven centiles 3rd, 10th, 25th, 50th (median), 75th, 90th, and 97th percentiles are commonly used. The percentile values increase as age grows. Each centile indicates what percentage of healthy children should have a

size that falls below that line. Third centile and 97th centile or −2SD or +2SD are commonly as cut-off points of normal growth. Children having the values within this range are referred to as normal while children under or above these limits are considered as potentially pathological. If a child is short (<3rd centile) or tall (>97th centile), the child should be examined thoroughly, and the heights of child's parents should be measured. If the child is within the normal limits of the parents, then the height is the most probably normal unless the parents have growth disorder or have growth in unfavorable conditions (Tanner 1990).

Head Circumference as a Biological Indicator

The HC is an anthropometric measurement of a child's head around its largest area. It is measured with a tape measure extending from the middle of the forehead to the farthest part in the rear of the head, as shown in Fig. 1. It measures the distance from above the eyebrows and ears and around the back of the head. It can be used to assess whether an infant's head size is within the normal range for age. The HC measurements at birth (together with weight and length) reflect intrauterine growth and allow for proper assessment of fetal growth. HC is also used as a proxy measure for brain development (both intrauterine and from birth) and can be predictive of some baby outcomes. HC has been long considered as a sensitive marker of neurodevelopment (Lipper et al. 1981). Hagan et al. (2007) recommend measuring HC eight times during the first 2 years of life. In many settings in low-income and middle-income countries (LMICs), however, the HC measurements are not performed regularly (Sindhu et al. 2019).

A Helsinki Birth Cohort Study (HBCS) has demonstrated that infancy, childhood, and adolescent periods are critical for the development of intellectual abilities, and slow HC growth during infancy may continue to appear in childhood and adolescents that extend widespread consequences on mental health throughout the lifespan (Räikkönen et al. 2009). A prospective study from the Southern India has shown HC to be positively correlated with learning and visuospatial ability in children aged 9 to 10 years (Veena et al. 2010).

Up to 3 years of age, head size reaches approximately 90% of the adult size and clinicians usually do not recommend the routine follow-up of the HC growth after this age for normal developing children. However, some neurological disorders and

Fig. 1 Measuring HC

genetic syndromes appear after 3 years of age among abnormally developing children. In addition, many medical problems, including various syndromes, may be related to microcephaly and macrocephaly. And more importantly, the HC measurement is a tool to assess the nutritional status of a child that could be normal, moderate underweight, severe underweight, moderate acute malnutrition (MAM), and severe acute malnutrition (SAM).

Construction of Growth Charts for HC: Different Methods

In 2007, Multicenter Growth Reference Study Group presented HC-for-age child growth standards for children from birth to 5 years old (WHO 2007). In 2000, Centers for Disease Control and Prevention (CDC) released the HC growth charts for the US children birth to 36 months of age (Kuczmarski et al. 2002). In 2007, WHO presented standard HC-for-age percentiles for boys and girls from birth to 60 months (see WHO 2007 and Fig. 2a, b).

After that some studies in different countries also reported the HC charts for monitoring head growth beyond the 36 months aged children, e.g., Rollins et al. (2010) established the HC growth reference charts for the US people aged birth to 21 years and Neyzi et al. (2015) established the HC reference values for the Turkish children, aged birth to 18 years.

However, the reference data on head size for the children of different developing countries is very limited. One study from Iran (Ayatollahi and Shayan 2006) presented the HC standards for the school children aged 6.5–11.5 years using a sample of 2237 children during the years 2002–2003, and another recent study (Aslam et al. 2019) from Pakistan presented the HC charts for children of 2 to 5 years. The recent study presented the percentile values (3rd to 97th) of HC and shows that centile curves were moving upward in both sexes with age. The boys had more increase in HC for the 3rd, 5th, and 50th percentiles than the girls had and for the upper percentiles, the similar patterns were also observed except for the 95th and 97th percentiles.

For computing the growth reference values, different statistical techniques have been applied by various researchers. As we know that measurements of growth-related variables like weight, HC, WC, skinfold thickness, etc. are highly dependent on a time covariate often age, and not often normally distributed. Usually, these measures have positively skewed (right-tiled) distributions. Therefore, the Z-scores and centiles could not be calculated from the mean and standard deviation, which assume a normal distribution. To handle this situation, Cole (1988) proposed a method that was named as the "Lambda-Mu-Sigma (LMS) method" and was used to create the growth centiles. This method assumes that the growth data at each age are normally distributed after applying a Box-Cox power transformation. The LMS method describes how the distribution changes by three uncorrelated smooth curves, L, M, and S curves. Of which, M (mu, the median curve) illustrates the trends in the median and the other two curves L (Lambda) and S (Sigma) represent the best power transformation needed to normalize the data and the coefficient of

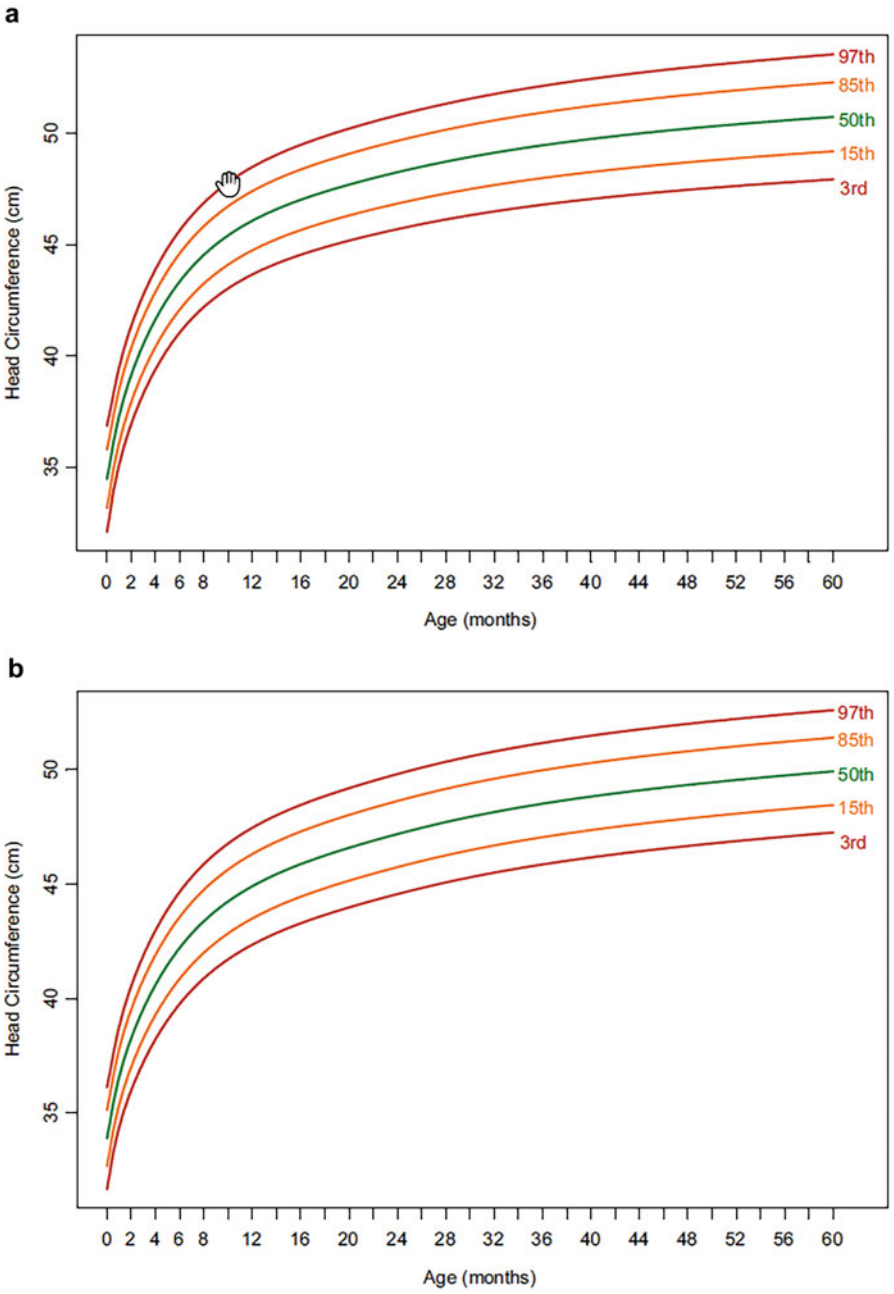

Fig. 2 (**a**) WHO head circumference-for-age percentiles for boys from birth to 60 months (WHO 2007) (**b**) WHO head circumference-for-age percentiles for girls from birth to 60 months (WHO 2007)

variation of the distribution at each age, respectively. One can use the GAMLSS package (Stasinopoulos and Rigby 2007) of R software for obtaining the smoothed centiles of this method. Different researchers used the LMS method for constructing the growth reference values, e.g., most recently, Asif et al. (2020) presented the growth reference curves of WC and WHtR for Pakistani children and adolescents aged 2–18 years using the LMS method. The same technique was also applied by Aslam et al. (2019) for establishing growth reference charts for HC of the Pakistani children (see Fig. 3a, b).

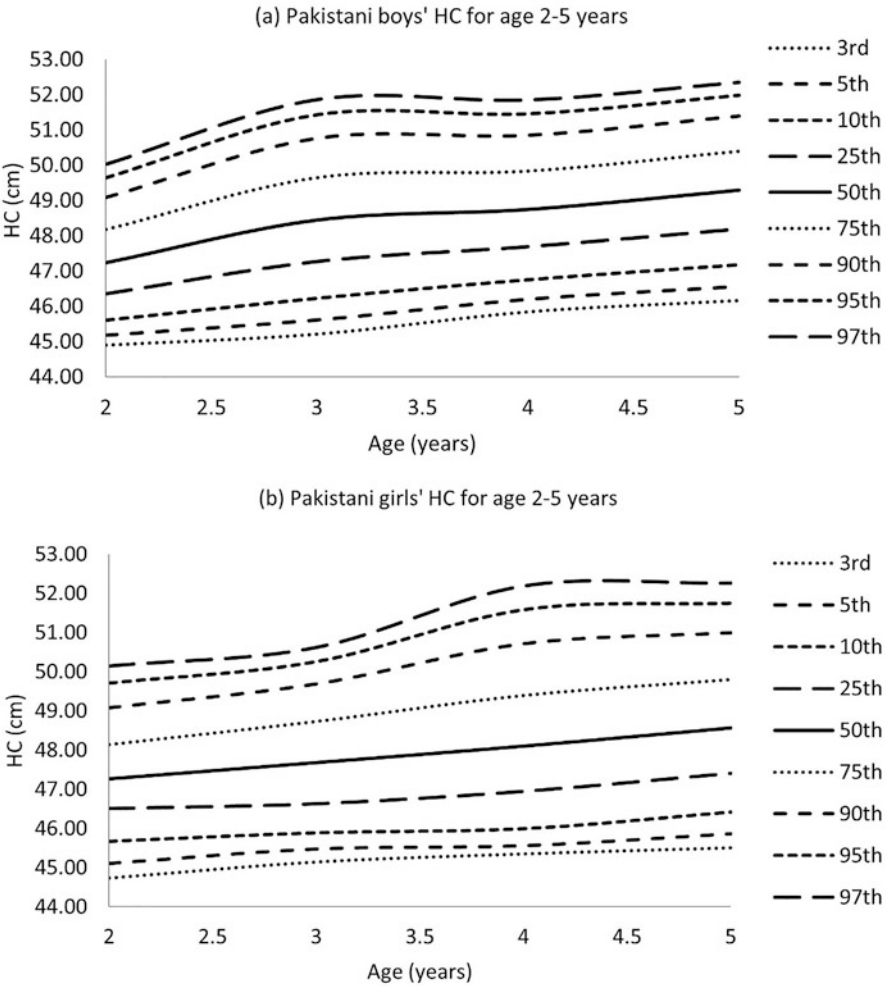

Fig. 3 (**a**) The HC growth charts for (2–5 years) Pakistani's boys (Aslam et al. 2019) (**b**) The HC growth charts for (2–5 years) Pakistani's girls (Aslam et al. 2019)

The quantile regression (QR), another more flexible statistical technique than the LMS technique, has also been applied for constructing the HC growth reference values. This statistical technique does not require any distributional assumption beforehand, and it is robust in response to outliers. It measures the effect of covariates not only in the center of the distribution but also in the upper and lower tails of the data set because of extremely low and extremely upper quantile are of our interest regarding growth charts. The details about this can be seen in earlier research. Chen (2005) firstly used the QR on national health and nutrition examination survey (NHANES) data for presenting the BMI growth charts of adults. The "rq" function which is available in the "QUANTREG" package, developed by Koenker et al. (2018) for the R software, can been used to implement the linear quantile regression model approach.

The Healy-Rasbash-Yang (HRY) method (Healy et al. 1988) is another distribution-free method for estimating age-related smoothed centiles for the HC. This method also makes no assumption about the nature of the measurement. Few Iranian researchers (for example, see Ayatollahi and Shayan 2006) used this method for presenting HC, height, and weight-related centile curves.

Assessment of Nutritional Status Using the HC

As discussed earlier, the nutritional status of children during infancy and early childhood has of paramount importance. Especially, after 6 months of age, children have some nutritional needs because of their growth and development. Many pediatric investigators (e.g., see Mandal and Bose 2010; Karabiber et al. 2001; Talebian et al. 2013 among many other) have attempted to use HC for monitoring under-nourishment and early diagnosis of neurological disorders among children.

WHO released a new international growth standard in 2007 showing how healthy children should grow (WHO 2007). The HC is measured according to percentiles that compare an individual's measurements with an appropriate age – and sex-specific growth chart. Percentile measurements are clinical indicators that rank the position of an individual's size and growth by indicating the percentage of the reference population an individual would equal or exceed. For example, 50% of the population are expected to be below the 50th centile; 90% below the 90th centile. One-half of all children at a given age are usually between the 25th and 75th centiles. Various definitions of normal limits are found in the literature. In Europe, the 3rd, 10th, and 25th centiles below the mean and the 75th, 90th and 97th centiles above it are used to determine cut-off points. The 5th and 95th centiles have been more routinely used in North America. WHO recommends the use of SD to determine normal limits. However, no clear cut-off point that defines abnormal growth has been identified; some researchers use 2 or 3 standard deviations as abnormal growth cut-off points when examining head circumference. Different growth charts (or curves) are applied to full-term and pre-term infants, respectively, because pre-term infants at birth are smaller in size than fetuses of the same gestational age (Olsen et al. 2010).

Table 1 The WHO (2007) recommended age- and sex-specific cut-off points for HC (cm)

	Boys		Girls	
Age (Years)	Moderate (−2SD)	Severe (−3SD)	Moderate (−2SD)	Severe (−3SD)
2	45.5	44.2	44.4	43.0
3	46.6	45.2	45.7	44.3
4	47.3	45.8	46.5	45.1
5	47.7	46.3	47.1	45.7

WHO (2007) recommended −2SD and −3SD of sex-specific cut-off points of HC for 2 to 5 years of age are given in Table 1. For instance, boys of 2 years of age having HC < −2SD values or <45.5 cm are classified as undernourished children. Among those having HC between −3SD to −2SD values or 44.2 cm to 45.5 cm are referred to as moderately undernourished and having HC < −3SD values or <44.2 cm are severely undernourished children.

In developing countries including Pakistan, pediatricians also used measurement of HC as a screening tool for undernutrition or its use in older children and adolescents (Asif et al. 2018a; Ayatollahi and Shayan 2006; Mandal and Bose 2010; Karabiber et al. 2001 etc.). A study by Asif et al. (2018b) including 1474 Pakistani children aged 2 to 5 years found that mean HC increased with advancement of age in both boys and girls and the nutritional status of the children was assessed using the following scheme (WHO 2007).

Applications to Prognosis

In addition to the use of the HC measures for the identification of child's nutrition status, it has also a great role in the other clinical examination. Many studies have also shown that serial HC measurements during early childhood is a robust reflector of the brain volume and can help to plot the trajectory of brain growth, thereby determining the cognitive functionality in later life (Bartholomeusz et al. 2002; Lindley et al. 1999).

According to Winter and Baraitser (1996), 114 syndromes associated with macrocephaly are listed. Some intrauterine infections may also lead to both microcephaly and macrocephaly. Many reports show that cognitive function, intracranial volume, and brain volume are closely related to the magnitude of HC. The clinical impact of HC measurements may become even more important. For proper interpretation of HC, pediatricians and neurologists frequently use the HC standard charts as a valuable tool for the brain developmental evaluation in children and for early diagnosis of neurological disorders.

Mini-Dictionary of Terms

- **Anthropometry.** It is mainly concerned with the measurement of physical sizes and shapes of human body and is used for understanding the human biological variability over time.

- **Body mass index (BMI).** It is measured as weight (in kg) divided by the height or recumbent length (in squared meters) of an individual.
- **Cut-off.** A designated limit beyond which a subject or observation is classified according to a pre-set condition.
- **Growth chart.** It is considered as an important tool that represents the growth pattern of different anthropometric measurements of a child in the form of reference curves.
- **Head circumference.** It is an anthropometric measurement of a child's head around its largest area. It is measured with a tape measure extending from the middle of the forehead to the farthest part in the rear of the head.
- **Z-score.** The deviation of a raw score (an individual value) from the mean (or median, usually used in anthropometry) value of a reference population, divided by the standard deviation of the reference population.

Key Facts of Human Growth

It is a numerical increase in size or mass of a human's body dimensions. It is a complex biological process that is regulated by genes and endocrines. Human growth is said to be normal when progression changes in growth variables are well-matched with the established standards for a given population. Human growth is age-related and occurs at different rates over different time periods. However, growth during infancy and childhood age, in all its aspects, is the priority concern to all.

Key Facts of Growth Reference Charts

A growth reference chart is considered as an important tool to represent the growth pattern of different anthropometric measurements of a child in the form of reference curves. Deviances from reference values may exhibit some problems in growth and need some further investigations. The first growth references were established in England in 1906.

Key Facts of Measuring HC

For infant and child growth monitoring, three most extensive and internationally recommended anthropometric indicators are weight-for-height, height-for-age, and weight-for-age. For the evaluation of nutritional status and other important clinical examinations of children of age 5 years or below, the HC measures have a vital role. The HC is also used for growth monitoring of the brain because cognitive functions, intracranial volume, and brain volume are closely related to the magnitude of HC.

Summary Points

- Growth reference chart is a great tool for monitoring the promotion of a child's health and nutrition.
- The measurement of HC has a crucial role in the pediatric growth and clinical examination for a child.
- Commonly, the Z-scores, percentiles, and percent of median are used to express the frequency distribution of the HC measurements in the growth charts.
- The popular methods to construct these charts are the LMS method and quantile regression.
- In addition to the use of the HC measures for the identification of child's nutrition status, it has also a great role in the other clinical examination.
- WHO (2007) recommended −2SD and −3SD of sex-specific cut-off points of HC for 2 to 5 years of age.
- The boys and girls of 2 years of age having HC less than 44.2 cm and 43.0 cm, respectively are referred to as severely undernourished children.

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Part VI

Biomarkers in Specific Conditions or Scenarios

Biomarkers of Malnutrition in Cirrhosis

34

Amir Gougol and Jaideep Behari

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Abstract

Malnutrition is highly prevalent in patients with cirrhosis and is associated with increased morbidity and mortality. As a modifiable risk factor, timely identification and appropriate management can potentially improve the outcomes in cirrhotic patients. Conventional tools to assess nutritional status such as BMI, plasma albumin, and prealbumin are influenced by the degree of liver dysfunction, limiting their accuracy in advanced cirrhosis. Among the studied serum biomarkers, branched-chain amino acids, certain adipokines such as leptin, and gastric hormones (ghrelin) were found as independent indicators of malnutrition. Vitamins and trace mineral deficiencies are common complications of malnutrition in cirrhosis and can serve as biomarkers of malnutrition. Novel approaches such as metabolomics and proteomics have recently evolved with promising accuracy. As alternative to serum biomarkers, anthropometric and radiologic tests to assess sarcopenia can serve as indirect measures to identify malnutrition in cirrhosis.

Keywords

Cirrhosis · Malnutrition · Energy expenditure · Albumin · Prealbumin · Branched-chain amino acids · Adipokines · Ghrelin · Micronutrients

Abbreviations

AAA	Aromatic amino acids
AASLD	American Association for the Study of Liver Diseases
BCAA	Branched-chained amino acids
BMI	Body mass index
CT	Computed tomography
EASL	The European Association for the Study of the Liver
GI	Gastrointestinal
HE	Hepatic encephalopathy
INR	International normalized ratio
LDUST	Liver Disease Undernutrition Screening Tool
NAFLD	Nonalcoholic fatty liver disease
PBC	Primary biliary cholangitis
RBP-4	Retinol-binding protein-4
REE	Resting energy expenditure
RFH-NPT	Royal Free Hospital Nutrition Prioritizing Tool
SIBO	Small intestinal bacterial overgrowth

Introduction

Malnutrition is a clinical state of nutrient deficiency resulting in major adverse outcomes in body function or composition (Lochs et al. 2006). Cirrhosis represents the late stage of chronic liver disease and is defined by distortion of hepatic architecture secondary to progressive hepatic fibrosis. In patients with cirrhosis, malnutrition is

very common, with prevalence reported up to 60% (Campillo et al. 2003; O'Brien and Williams 2008; Tai et al. 2010). The connection between cirrhosis and malnutrition is bidirectional; cirrhosis can cause diminished appetite, poor oral intake due to encephalopathy, and attenuated absorption leading to malnutrition. On the other side, malnutrition accelerates hepatocyte injury and increases the risk of cirrhosis complications such as hepatic encephalopathy and superimposed infection. Furthermore, malnutrition enhances generalized skeletal muscle loss, a condition referred to as *sarcopenia* (Hanai et al. 2016; Cruz-Jentoft et al. 2019). This results in diminished physiologic reserve, poor functional performance, and vulnerability to stressors, generally referred to as *frailty* (Morley et al. 2013). Although malnutrition, sarcopenia, and frailty are separate entities, they are closely interrelated and usually coexist. Hence, tools to identify sarcopenia and frailty can be used as alternative methods to assess malnutrition.

Poor nutritional status is associated with increased morbidity and mortality in patients with cirrhosis (O'Brien and Williams 2008). Pretransplant malnutrition is also a predictor of poor surgical outcomes and postoperative complications (Kaido et al. 2010). Furthermore, malnutrition closely associates with sarcopenia and frailty, which are major determinants of poor quality of life, outcomes after transplantation, and overall mortality in cirrhosis (Lai et al. 2014, 2018; Derck et al. 2015; Dunn et al. 2016, 2020). Impaired nutritional intake is a modifiable risk factor, and early identification can potentially alter clinical outcomes of cirrhosis. Therefore, timely diagnosis, appropriate intervention, and monitoring the response to nutritional therapies are essential in cirrhosis management. Based on these evidences, both the American Association for the Study of Liver Disease (AASLD) and the European Association for Study of the Liver (EASL) recommend that all patients with cirrhosis should be assessed for malnutrition, both at the time of diagnosis and longitudinally during the management of cirrhosis.

Screening and assessment of nutritional status in patients with advanced liver disease are challenging. Simple anthropometric measurements such as weight and body mass index (BMI) may not be accurate due to the frequent presence of ascites and volume overload in advanced liver disease (O'Brien and Williams 2008). Conventional laboratory biomarkers of malnutrition such as plasma proteins are ambiguous in face of cirrhosis because their synthesis or excretion depends on hepatocyte function (Figueiredo et al. 2000). Consequently, further serologic, anthropometric, and radiologic measures were developed to optimize accuracy of malnutrition assessment in cirrhosis. Recently, with the advent of computational biology, novel techniques such as metabolomics and proteomics evolved to optimize the detailed assessment of malnutrition in cirrhosis. In this chapter, we aim to review the objective parameters to identify malnutrition in patients with cirrhosis.

Mechanisms of Malnutrition in Cirrhosis

Reduced Oral Intake

Anorexia is a common complication of cirrhosis, reported in up to 90% of patients with advanced liver disease (Achord 1987). In the late stages of cirrhosis, patients can experience impairment in smell and taste, which contributes to poor oral intake.

Zinc deficiency can aggravate anorexia and intensify deficiency of macro- and micronutrients including zinc (Johnson et al. 2013). Interruption of regular oral intake frequently occurs in patients with decompensated cirrhosis due to recurrent episodes of hepatic encephalopathy, hospitalizations, and need for fasting before procedures (Bunchorntavakul and Reddy 2020). Subsequently, oral intake can be greatly impacted. Dietary restrictions recommended by clinicians, such as sodium and fluid restriction, can have detrimental effect on protein and essential nutrients. Nutritional education tailored to the patient dietary style should be coupled with dietary restrictions to minimize the risk of malnutrition.

Increased Metabolic Demand

Chronic liver disease may lead to poor synthesis and eventually depleted stores of glycogen in hepatocytes. In the late stage of cirrhosis, a short period of fasting such as an overnight fast can deplete limited glycogen stores. Next, the body utilizes alternative sources of glucose such as amino acids and glycerol, resulting in muscle and fat tissue wasting. Therefore, patients with cirrhosis require frequent repletion of nutrients to meet the metabolic need (Eghtesad et al. 2013). Furthermore, patients with cirrhosis exhibit a hypermetabolic state, with resting energy expenditure (REE) of about 20% more than expected. Hypermetabolism can result from the increased beta-adrenergic activity and persistent cytokine activation (Prieto-Frías et al. 2016).

Malabsorption

Impaired enterohepatic bile acid circulation can exist in patients with chronic liver disease, particularly cholestatic liver disease such as primary biliary cholangitis (PBC). Fat digestion may be impaired due to insufficient bile acid secretion in the gastrointestinal (GI) lumen, leading to poor absorption of long-chain fatty acids and fat-soluble vitamins (Venu et al. 2013). GI tract motility can also be diminished in chronic liver disease and impact normal GI tract microbiota. Dysmotility can be mediated by increased nitric oxide, sympathetic nervous system hyperactivation, and limited physical activity in cirrhotic patients. Dysmotility, in turn, can predispose small intestinal bacterial overgrowth (SIBO), a condition identified in up to half of patients with cirrhosis (Bauer et al. 2001). Both dysmotility and SIBO limit the absorption capacity of the GI tract. Figure 1 summarizes the mechanisms contributing to the development of malnutrition in cirrhosis.

Clinical Assessment

Nutritional Questionnaires

Multiple subjective screening tools have been designed to screen for malnutrition. Royal Free Hospital Nutrition Prioritizing Tool (RFH-NPT) and Liver Disease Undernutrition Screening Tool (LDUST) were studied in patients with chronic

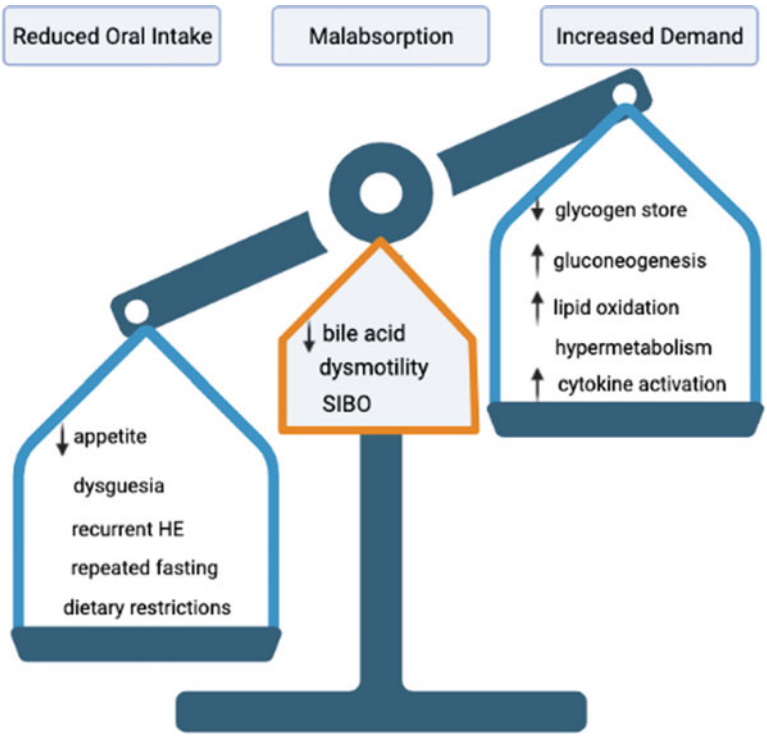

Fig. 1 Mechanisms of malnutrition in cirrhosis. HE, hepatic encephalopathy; SIBO, small intestinal bacterial overgrowth

liver disease. RFH-NPT is a stepwise screening tool that categorizes the patients into mild, moderate, and high risk of malnutrition. A study showed RFH-NPT as an independent predictor of hepatic decompensation and transplant-free survival in cirrhosis (Borhofen et al. 2016). However, RFH-NPT had low negative predictive value (37%), questioning its utility as a screening tool (Booi et al. 2015). Both RFH-NPT and LDUST scores are based on BMI and weight loss, which can be influenced by the presence of ascites, volume overload, and diuretic use in patients with advanced liver disease (Traub et al. 2020). Therefore, the reliability of RFH-NPT and LDUST as independent markers of malnutrition is uncertain, highlighting the need for objective methods to identify malnutrition in cirrhosis.

Energy Expenditure

Assessment of total energy expenditure is essential to estimate nutritional requirements. Multiple methods have been developed to calculate REE, which represents 50–75% of total body energy requirement. There are two approaches to assess REE: (1) predictive models and (2) indirect calorimetry. Predictive models such as the Harris-Benedict equation estimate REE based on a combination of demographic and

anthropometric data such as weight and BMI. Predictive models significantly underestimate the energy requirement in patients with cirrhosis (Madden and Morgan 1999). Therefore, EASL guidelines recommended measurement of REE obtaining from indirect calorimetry. Indirect calorimetry estimates the amount of energy generated by measuring oxygen consumed and carbon dioxide produced. Indirect calorimetry was traditionally performed using a large metabolic cart with ventilator hood, thus limiting its feasibility in routine clinical care (Plauth et al. 2009). Handheld calorimeters were recently developed facilitating bedside or outpatient use.

Laboratory Evaluation

Plasma Proteins

Generally, plasma proteins such as albumin, prealbumin, transferrin, and prothrombin are the most commonly used biomarkers of nutritional status in patients with chronic illness (Fuhrman et al. 2004). Yet, these proteins are mainly synthesized and secreted by the liver, and their levels may correlate with the degree of liver dysfunction rather than malnutrition. While these plasma proteins may still be helpful biomarkers of nutritional status in early cirrhosis, their reliability in advanced stages of cirrhosis is questionable.

Albumin

Albumin is exclusively synthesized by hepatocytes and performs multiple vital functions in the body such as maintenance of colloid osmotic pressure, ligand binding, and molecular transport. Albumin has a half-life of approximately 20 days, which makes it a marker of long-term nutritional status. Albumin is one of the main determinants of liver disease severity and frequently applied in prognostic models such as the Child-Pugh classification. Studies comparing different nutritional biomarkers with anthropometric tests such as mid-arm circumference (MAC) found albumin has poor specificity and low positive predictive value to detect malnutrition in the late stages of cirrhosis (Piquet et al. 2006). While hypoalbuminemia can still be used as a marker of malnutrition in the early stages of cirrhosis, interpretation in advanced liver disease is challenging.

Prealbumin

Prealbumin is another plasma protein commonly used as a marker of nutritional status in multiple clinical settings. Compared to albumin, prealbumin has a shorter half-life (about 2 days). Thus, prealbumin can reflect acute changes in nutritional status. In cirrhosis, a lower level of prealbumin is associated with a higher rate of complications such as hepatic encephalopathy (Alvares-da-Silva and Reverbel da Silveira 2005; Tan et al. 2019). Yet, the specificity of prealbumin to assess for malnutrition in cirrhosis is limited. Like albumin, prealbumin is mainly synthesized by hepatocytes, making it a less reliable marker of malnutrition in advanced cirrhosis. In addition, prealbumin is a negative acute-phase reactant; thus, the source of

low level is ambiguous in settings of acute inflammation or superimposed infection (Beck and Rosenthal 2002).

Transferrin

Transferrin is another plasma protein that has been frequently used to assess nutritional status. The half-life of transferrin is 10 days. Transferrin is also synthesized in the liver, and its level can be decreased in the late stages of cirrhosis. Transferrin has a key role in iron transport, and the level is elevated in iron deficiency anemia (Bharadwaj et al. 2016).

Retinol-Binding Protein-4

Serum retinol-binding protein-4 (RBP-4) is a complex protein acting as a specific transporter of retinol (vitamin A). RBP-4 is synthesized in both adipose tissue and the liver and excreted by the kidneys. Rapid turnover makes it a better predictor of recent changes in nutritional status. Studies have shown reduced RBP-4 level is associated with weight loss and adipose tissue depletion (Haider et al. 2007). Evaluating patients with severe malnutrition who receive parenteral nutrition showed RBP-4 levels rise quickly, suggesting that it could be used as a biomarker to assess response to nutritional therapies. Yet, RBP-4 specificity is limited in advanced liver disease and chronic kidney disease, as they are the source of synthesis and excretion, respectively (Chaves et al. 2015).

Branched-Chained Amino Acids

Branched-chained amino acids (BCAA), including valine, leucine, and isoleucine, are a group of essential amino acids, i.e., they must be supplied from a nutritional source. BCAA are constituent of key plasma proteins such as albumin. They are also the precursor of glutamate, which is a central factor in ammonia detoxification. Deficiency of BCAA is common in patients with cirrhosis, resulting from the combination of poor nutritional intake, hypermetabolism, and excessive use for ammonia detoxification (Yamato et al. 1995). On the other hand, aromatic amino acids (AAA) such as phenylalanine, tyrosine, and tryptophan are elevated in advanced liver disease likely resulting from portosystemic shunt (Okuno et al. 1995). Reduced BCAA/AAA ratio is associated with increased incidence of hepatic encephalopathy, as well as mortality (Als-Nielsen et al. 2003). BCAA/AAA ratio is independent of cirrhosis severity; therefore, it can act as a reliable indicator of nutritional deficiency in cirrhosis (Holecek 2015). A clinical trial suggested a beneficial effect of BCAA supplementation in cirrhosis (Kawaguchi et al. 2011). Subsequently, the American Society for Parenteral and Enteral Nutrition (ASPEN) recommended BCAA supplementation in patients with refractory hepatic encephalopathy (Force 2002).

Adipokines

Adipokines are polypeptides secreted mainly by adipose tissue that regulate several metabolic pathways in systemic lipid metabolism (Marra and Bertolani 2009).

Adipokines play a significant role in controlling appetite, energy expenditure, glucose uptake, and body fat storage. Thus, their serum levels can indirectly represent nutritional status.

Leptin

Leptin is secreted from adipose tissue and regulates the function of the hypothalamus by inhibiting appetite and enhancing energy expenditure. Leptin levels are proportional to total body fat composition, so a reduced level can represent depleted fat stores, which is a consequence of malnutrition. A significant decrease in leptin level was associated with malnutrition in several malignancies and chronic pulmonary disease (Takabatake et al. 2001; Wallace et al. 2002). A prospective study evaluating multiple biomarkers of malnutrition in cirrhosis found threefold reduced leptin level in malnourished subjects. The leptin level was independent of disease severity, suggesting it may have clinical value as a reliable biomarker of nutritional status in patients with advanced liver disease (Rachakonda et al. 2016).

Adiponectin

Adiponectin is an adipokine with several actions in carbohydrate and lipid metabolism (Silva et al. 2014). The adiponectin level increases in response to body fat depletion, explaining its role as an indicator of malnutrition (Lee et al. 2011). However, serum levels of adiponectin have contradictory correlation with liver disease based on the degree of hepatic dysfunction. Adiponectin stimulates glucose uptake, and fatty acid oxidation by skeletal muscles, resulting in decreased insulin sensitivity and triglyceride level. Adiponectin can act as a hepatoprotective agent by reversing hepatic steatosis in nonalcoholic fatty liver disease (NAFLD). Reduced level of adiponectin is associated with the progression of non-cirrhotic NAFLD (Kim et al. 2005). By contrast, adiponectin level is elevated in the late stages of cirrhosis, which can be explained by the fact that hepatocytes are a major source of its degradation and excretion (Tietge et al. 2004).

Resistin

Resistin is an adipokine secreted by white adipose tissue and mononuclear cells (Liakopoulos et al. 2006). Resistin is involved in the regulation of glucose and free fatty acid metabolism and may contribute to the development of obesity and insulin resistance. Serum resistin also correlates with the risk of hepatic steatosis (Bajaj et al. 2004). In patients with cirrhosis, the resistin level is elevated, proportional to disease severity (Ajmera et al. 2017). Further studies are warranted to elucidate the accuracy of resistin to identify nutritional status at different stages of chronic liver disease.

Plasma Ghrelin

Ghrelin is a hormone secreted by the stomach and stimulates appetite and growth hormone release by the hypothalamus. Plasma ghrelin plays a major role in

controlling feeding behavior (Tacke et al. 2003). In patients with cirrhosis, plasma ghrelin is positively correlated with BMI, MAC, and triceps skinfold thickness. The level of ghrelin is not affected by the severity of liver disease, which makes it an objective indicator of oral intake in this group of patients (Takahashi et al. 2006).

Micronutrients

Malabsorption of micronutrients is relatively common in cirrhosis, which can result from Bile salt deficiency, bacterial overgrowth, and portal hypertensive changes of the small intestine (Cheung et al. 2012). Prevalence of certain micronutrient deficiencies varies based on etiology of cirrhosis.

Fat-Soluble Vitamins (Vitamins A, D, E, and K)

Reduced intraluminal bile acids in chronic liver diseases, particularly cholestatic liver disease such as PBC, predispose to malabsorption of fat-soluble vitamins. The mechanism of fat-soluble vitamin deficiency is multifactorial, including poor nutritional intake and impaired hepatic utilization; thus, their deficiency is not limited to cholestatic liver disease. A study by Fisher et al. showed a high prevalence fat-soluble vitamin deficiency in cirrhosis with no significant difference in prevalence based on the etiology of cirrhosis (Fisher and Fisher 2007). Based on these data, EASL recommends screening and monitoring of vitamins A, D, and E and INR (derived from prothrombin time, which is elevated in vitamin K deficiency) in patients with cholestatic liver disease and routine vitamin D screening in all patients with cirrhosis.

Vitamin D deficiency is the most common micronutrient deficiency in cirrhosis, reported in up to 90% of patients. About one-third of cirrhotic patients suffer from severe vitamin D deficiency (Arteh et al. 2010). The presence of vitamin D deficiency is independent of the etiology or severity of chronic liver disease (Venu et al. 2013). Vitamin D deficiency increases the risk of osteoporosis and sarcopenia in cirrhosis (Konstantakis et al. 2016). Vitamin A deficiency is the second most common vitamin deficiency in cirrhosis, with small case series reporting prevalence of up to 60%. As routine screening for vitamin A deficiency is not universal, its burden may be underestimated. Vitamin A deficiency can result in subtle clinical symptoms such as night blindness, which may not be detected unless the patients are evaluated by visual testing (Abbott-Johnson et al. 2011).

Water-Soluble Vitamins

Deficiencies of vitamin B1 (thiamine), vitamin B6 (pyridoxine), vitamin B12, and folic acid are common in alcoholic liver disease and can result in neurologic disturbances such as peripheral neuropathy (Rossi et al. 2015). On the contrary, vitamin B12 levels may be paradoxically elevated in conditions such as viral hepatitis and hepatocellular carcinoma in the setting of hepatocyte cytolysis releasing vitamin B12 stores (Ermens et al. 2003).

Minerals

Zinc, magnesium, and selenium deficiencies are frequently identified in cirrhosis. Trace mineral deficiency is aggravated in patients requiring diuretics therapy (Chiba et al. 2013). Zinc plays a key role in ammonia detoxification; thus, deficiency is associated with the progression of hepatic encephalopathy (Sengupta et al. 2015). Zinc deficiency is also associated with diminished taste resulting in aggravation of generalized malnutrition in cirrhosis (Kodama et al. 2020).

Emerging Biomarkers

With the rapid progression of molecular techniques and computational biology, complex analyses and interpretation of large-scale molecular patterns have become possible. These techniques enable researchers to analyze a large number of biological factors to discover distinct phenotypes associated with physiologic and pathologic conditions. Genomics, metabolomics, proteomics, and lipidomics have been studied as novel biomarkers to increase precision of clinical assessment.

Metabolomics

Metabolomics is a strategy to analyze hundreds to thousands of metabolites to depict detailed phenotypes of metabolic pathways. In chronic liver disease, multiple metabolic pathways are impaired. Hence, metabolomics is an innovative strategy to delineate pathologic processes and provide distinct biomarkers (Rachakonda et al. 2014). In a recent study, 747 metabolites were analyzed to determine the metabolic pathways specific to malnutrition in patients with decompensated cirrhosis. Serum metabolomic profiles characterized by reduced sphingolipids were significantly associated with malnutrition in cirrhosis. Furthermore, metabolic profiling of malnutrition was independent of liver disease severity (Rachakonda et al. 2019).

Proteomics

Proteins are responsible for the structure, function, and growth of cells. Serum proteins exhibit a high level of diversity with varying quantities in different physiological conditions. Although the human genome has been fully sequenced, determination of the human proteome is an ongoing project. A large-scale study of proteins using complex bioinformatics models to map physiologic and pathologic processes is referred to as proteomics (Kavallaris and Marshall 2005). Hepatocytes synthesize the majority of serum proteins in a very complex regulated fashion; thus, proteomics can serve as groundbreaking biomarkers to delineate metabolic pathways in cirrhosis (Kuscuoglu et al. 2018). Proteomic profiling techniques have been studied to assess the severity of liver disease and predict the risk of complications and hepatocellular carcinoma (Miller et al. 2014; Nallagangula et al. 2018). Proteomic analysis was found to be an accurate marker of malnutrition in certain chronic illness as well (Gonzales et al. 2020). While further studies are warranted, proteomics is a promising approach to identify novel biomarkers of malnutrition in cirrhosis.

Ancillary Tests

Although malnutrition, sarcopenia, and frailty are separate entities, they are closely interrelated and usually coexist. Hence, tools to identify sarcopenia and frailty can be used as alternative methods to assess malnutrition. Figure 2 illustrates the association between malnutrition, sarcopenia, and frailty in cirrhosis patients.

Anthropometric Tests

Anthropometric tests are techniques to assess lean body mass, which can be performed at the bedside. Given the high prevalence of ascites, and body fluid overload, weight and BMI are not reliable indicators of sarcopenia; thus, specific tests such as MAC and skinfold thickness are used in cirrhosis. Tests assessing frailty can also serve as surrogate markers of malnutrition. A study evaluating multiple anthropometric and frailty tests found MAC and handgrip strength as the most sensitive markers of malnutrition in cirrhosis (Figueiredo et al. 2000).

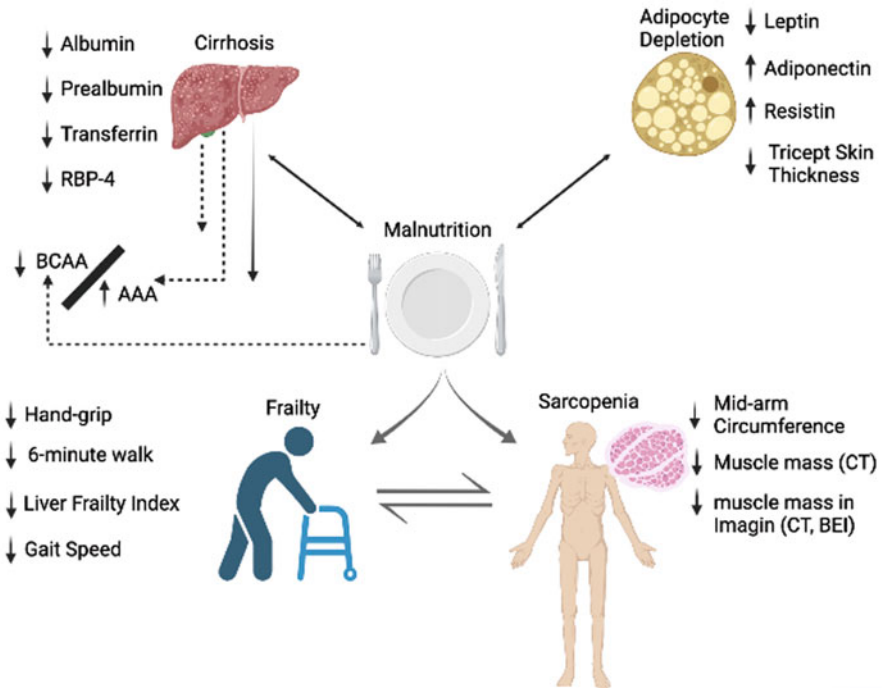

Fig. 2 Association of malnutrition with cirrhosis, lipid metabolism, sarcopenia, and frailty. RBP-4, retinol-binding protein-4; CT, computed tomography; BEI, bioelectrical impedance

Radiologic Assessment

Radiologic evaluation of sarcopenia has been studied by bioelectrical impedance, and computed tomography (CT) was applied as a tool to quantify sarcopenia in patients with cirrhosis. CT scan is the gold standard test to quantify skeletal muscle loss, but its routine use in a repeated fashion is impractical given the issues with availability, cost, and risk of radiation (Carey et al. 2019).

Conclusion

Malnutrition is a very common complication of cirrhosis and impacts morbidity and mortality. Therefore, evaluation and management of malnutrition is a key component of cirrhosis management. Conventional methods such as BMI and plasma proteins are not accurate in assessing malnutrition in cirrhosis. BCCA, serum leptin, and ghrelin were found to be potential biomarkers of malnutrition in cirrhosis (Table 1). Micronutrient deficiency is common and should be assessed routinely. Metabolomic and proteomic profiling are promising techniques that in the future may lead to the discovery of novel, objective biomarkers of cirrhosis-associated malnutrition. Methods to assess sarcopenia such as anthropometric tests and CT scans can be used as an indirect marker of malnutrition as well.

Applications to Prognosis

Malnutrition is independently associated with increased morbidity and mortality in cirrhosis. As a modifiable risk factor, early diagnosis and management of malnutrition can decrease complications and subsequently overall mortality. In this chapter, we reviewed several serum biomarkers of malnutrition, which can potentially act to increase accuracy of prognostication in patients with cirrhosis. Furthermore, we discussed the tests to screen sarcopenia, a common complication of malnutrition, which has an important prognostic role in patients with chronic liver disease.

Mini-dictionary of Terms

- **Adipokines**: A group of polypeptides secreted mainly by adipose tissues and are involved in controlling systemic lipid metabolic pathways.
- **Cirrhosis**: The late stage of chronic liver disease and is defined by the distortion of hepatic architecture secondary to progressive hepatic fibrosis.
- **Essential amino acids**: A group of amino acids that cannot be synthesized by the body and have to be obtained from diet.
- **Frailty**: A state of diminished physiologic reserve, poor functional performance, and vulnerability to stressors.

Table 1 Serum biomarkers of malnutrition in cirrhosis

Category	Factors	Change with severity of cirrhosis	Conditions associated with lower level	Conditions associated with higher level	Practical comments
Plasma proteins	Albumin	Decreased	Infection Acute and chronic inflammation Nephrotic syndrome	Dehydration Steroid use	Extended half-life
	Prealbumin	Decreased	Anemia Surgery Infection Acute inflammation	CKD Hyperthyroidism Pregnancy	Short half-life, indicator of acute changes
	Transferrin	Decreased	Nephrotic syndrome Inflammation	Iron deficiency anemia Pregnancy OCP use	Short half-life
	Prothrombin	Decreased (PT increased)	DIC Antiphospholipid syndrome		
	Retinol-binding protein-4	Decreased (increased in early stages of NASH)	Vitamin A deficiency Hyperthyroidism Infection	CKD Insulin resistance	

(continued)

Table 1 (continued)

Category	Factors	Change with severity of cirrhosis	Conditions associated with lower level	Conditions associated with higher level	Practical comments
Amino acids	BCAA/AAA ratio	No change			Associates with HE
Adipokines	Leptin	No change	Anorexia nervosa Low GnRH	Pregnancy Childhood	
	Adiponectin	Increased	Obesity Diabetes Hyperlipidemia		
	Resistin	Increased		Diabetes mellitus	
Hormones	Ghrelin	No change	Gastric bypass surgery	Diabetes mellitus Hypertension CKD Prader-Willi syndrome	

Abbreviations: CKD, chronic kidney disease; OCD, oral contraceptive use; PT, prothrombin time; DIC, disseminated intravascular coagulation; NASH, nonalcoholic steatohepatitis; BCAA, branched-chained amino acids; AAA, aromatic amino acids; GnRH, gonadotropin-releasing hormone

- **Malnutrition**: Clinical state of nutrient deficiency resulting in major adverse outcomes in body function or composition.
- **Metabolomics**: An approach to analyze hundreds to thousands of metabolites to depict detailed phenotypes of metabolic pathways.
- **Micronutrients**: Nutrients, encompassing vitamins and minerals, that are required in small quantities by the body but are essential for optimum health.
- **Proteomics**: An approach to study a large number of polypeptides with computational analysis to determine the function and structure of the organism.
- **Resting energy expenditure**: The calories required over a 24-h period by the body during a non-active period.
- **Sarcopenia**: A condition of generalized skeletal muscle loss secondary to systemic illness.

Key Facts

Key Facts of Malnutrition in Cirrhosis

- Malnutrition is reported in 60–90% of cirrhosis patients.
- Malnutrition commonly associates with sarcopenia and frailty.
- Malnutrition is a modifiable risk factor.
- Malnutrition increases morbidity and mortality in cirrhosis.

Key Facts of Biomarkers of Malnutrition in Cirrhosis

- Conventional biomarkers of nutrition lack specificity in cirrhosis.
- Essential amino acid deficiency is a predictor of hepatic decompensation.
- Adipokines regulate the relationship between the liver and body fat and can act as a biomarker of malnutrition.
- Hormones that affect feeding behavior can be used as biomarkers of nutritional status.
- Vitamin and trace mineral deficiencies are very common in cirrhosis, with their prevalence varying depending on the etiology of liver disease.
- Emerging techniques such as metabolomics and proteomics have the potential to reveal novel biomarkers of malnutrition in cirrhosis.

Summary Points

- Subjective nutritional assessments are not sensitive in cirrhosis.
- Energy expenditure should be measured by indirect calorimetry when available.
- Albumin and prealbumin are influenced by the degree of liver disease and are not reliable biomarkers of malnutrition in the late stages of cirrhosis.

- Branched-chain amino acid deficiency is a complication of malnutrition and predicts the risk of hepatic encephalopathy.
- Leptin level is reduced in malnourished patients with cirrhosis, independent of cirrhosis severity.
- Plasma ghrelin modifies appetite and can serve as an objective marker of feeding behavior.
- Vitamin D deficiency exists in up to 90% of cirrhosis patients and is associated with osteoporosis, sarcopenia, and prognosis.
- Zinc deficiency associates with decreased appetite and a higher rate of hepatic encephalopathy.
- Metabolomics and proteomics are groundbreaking techniques that may increase precision in the diagnosis of malnutrition in cirrhosis through discovery of novel serum biomarkers.

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Inflammatory Biomarkers in Nutrition

35

Impact of Diet

Sandra Abreu and Mário Sousa-Pimenta

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Abstract

Chronic inflammation triggers the development of a wide range of diseases such as cardiovascular disease, diabetes, cancer, and neurodegenerative pathologies, among others. In this context, numerous biomarkers have been used to monitor or

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evaluate the inflammatory process in humans. Apart from other factors, diet or its components may influence the inflammatory process. The main aims of this chapter were to review the biomarkers related to chronic inflammation and the current evidence on the impact of diet on inflammation. The C-reactive protein, interleukin-6, and tumor necrosis factor alpha are the biomarkers most frequently used in nutritional studies. Although findings are still inconsistent, mainly due the high heterogeneity across studies, nutrients (n-3 polyunsaturated fatty acids, vitamins C and E, magnesium, flavonoids), foods (fruits, vegetables, dairy products, olive oil, nuts and seeds), and healthy dietary patterns (Mediterranean diet, anti-inflammatory diet, plant-based diets) seemed to have a beneficial effect on inflammatory biomarkers.

Keywords

Inflammation · Cytokines · Adipokines · Fatty acids · Vitamin C · Vitamin E · Magnesium · Fruits · Vegetables · Dairy products · Dietary patterns

Abbreviations

CRP	C-reactive protein
DASH	Dietary Approaches to Stop Hypertension
DHA	Docosahexaenoic acid
DII	Dietary Inflammatory Index
EPA	Eicosapentaenoic acid
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
MCP-1	Monocyte chemotactic protein-1
MedD	Mediterranean diet
MUFA	Monounsaturated fatty acids
PAI-1	Plasminogen activator inhibitor 1
PPAR-γ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acids
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RCT	Randomized controlled trials
ROS	Reactive oxygen species
SFA	Saturated fatty acids
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1

Introduction

The twentieth century represented a milestone in the history of humankind. We witnessed a progressive and continuous increase of human longevity accompanied by a decrease in deaths associated with infectious diseases (such as pneumonia, tuberculosis, and influenza), albeit with a modest increment in those derived from diseases resulting from slow damage accumulation, namely, cardiovascular, cerebrovascular, and neoplastic diseases (Jones et al. 2012).

The slow damage accumulation diseases of the century share some common molecular and cellular features, with inflammation being regarded as one of the most prominent. Over the aging process, the exposure to pathogen-associated molecular patterns, to nutrients and/or its metabolites, and to danger-associated molecular patterns will drive the activation of the immune system, pruning the subject to a pro-inflammatory state. The frequency, intensity, and directionality of the immune response will determine the short- and long-term consequences of this biological process. Chronic pro-inflammatory stimuli and milieu will potentiate the genesis of cardiometabolic, neoplastic, and neurodegenerative disorders. In this regard, the inflammation related to senescence (low-grade inflammation occurring during aging) will share and overlap with some molecular and cellular clues of metaflammation resulting from metabolic disorders, with lifestyle and diet being highlighted as potential environmental modulators of this phenomenon (Franceschi et al. 2018).

Taken altogether, the inflammation triggered by external stimuli (diet or exposure to pathogens) or as a direct consequence of biological senescence will foster disease development, such as asthma, diabetes, cardiovascular diseases, and cancer. As such, the modification of these health conditions is of particular interest to researchers. Over the past few decades, several epidemiological studies have investigated the effect of diet on markers of inflammation (Calder et al. 2011; Barbaresko et al. 2013; Hart et al. 2021). It has been described that diet may modulate inflammation through a wide range of mechanisms including, but not limited to, lowering the production of inflammatory mediators, decreasing reactive oxygen species (ROS) production, and promoting the function of gut microbiome and the anti-inflammatory response (Calder et al. 2009).

In this chapter, we present the biomarkers related to a chronic pro-inflammatory state and its surrogate hallmarks in impaired nutritional status (ROS production, fatty acid metabolism derivatives, insulin resistance, immune dysfunction or reprogramming, and adipokine secretion pattern) as well as review evidence on the impact of diet on inflammatory biomarkers in human studies. Some specific nutrients, isolated foods or food groups, and dietary patterns will be discussed, such as fatty acids, vitamins C and E, magnesium, fruits and vegetables, dairy products, and a priori and a posteriori dietary patterns.

Biomarkers of Inflammation

The mediators implicated in the inflammatory cascade initiation display an imbricate relation and crossregulate each other, not infrequently perpetuating their actions and disrupting the general homeostasis which leads to disease burden.

ROS act as cell signaling modulators, and their generation and catabolism play a major role in the phagocyte system adjudicated to the innate immune system response. On the other hand, when prooxidant and antioxidant signaling becomes unbalanced, there is a propensity to physiological dysfunction and disease development. For instance, interaction of some ROS with phospholipids in cell membranes will foster the lipid peroxidation, herein perpetuating the cellular damage and inflammatory response. Also, protein oxidation can impair several metabolic processes and disrupt cell signaling pathways, while deoxyribonucleic acid oxidation is associated with increased risk of strand breaks and accelerated senescence (Auten and Davis 2009). Moreover, the redox signaling and balance are also widely implicated in the immunological response's modulation. In physiological levels and accordingly to the surrounding microenvironment and antigen stimulation of T cell receptor, ROS will modulate the expression of transcription factors implicated in T cell fate. Indeed, albeit ROS are essential in the initiation of inflammatory responses, a metabolic fine-tuning is mandatory because an imbalance in redox state may be deleterious, impairing adequate host defense responses (Rashida Gnanaprakasam et al. 2018).

Fatty acids, for instance, rather than sources of energy are a structural component of the cell membrane and precursors of signaling molecules that play a key role in a wide spectrum of biological processes. The n-3 polyunsaturated fatty acids (PUFA) present in fish oil, as docosahexaenoic acid (DHA) and eicosapentahenoic acid (EPA), for example, are precursors of anti-inflammatory lipid mediators (Fritsche 2015). Arachidonic acid, on the other spectrum, is widely available in meat and can also be incorporated in the cell membranes subsequently leading to accumulation of pro-inflammatory eicosanoids such as prostaglandin-E2 (dependent upon cyclooxigenases activity and responsible for vasodilation and increased vascular permeability) and leukotriene B4 (in a reaction catalyzed by 5-lypoxigenase and acting as a chemoattractant for neutrophils) (Calder 2011).

Lipotoxicity is associated with central/visceral adipose tissue deposition and leads to a chronic subclinical inflammation that will foster the development of insulin resistance, ultimately representing a state of reduced responsiveness to normal circulating levels of insulin. In the context of disrupted fatty acid metabolism (increased uptake or decreased oxidation), long-chain fatty acids and diacylglycerol accumulate in the skeletal muscle and liver triggering the activation of protein kinases. These will phosphorylate the insulin receptor substrate 1 (IRS-1) in serine/threonine domains, herein reducing the ability of insulin to stimulate IRS-1 tyrosine phosphorylation (and IRS-2 tyrosine phosphorylation in the liver) and activate phosphatidylinositol 3-kinase, thereby hampering glucose uptake and subsequent metabolization (Savage et al. 2005). Simultaneously, a chronic pro--inflammatory state is also known to endeavor insulin resistance, with well-known contributions of interleukin-6 (IL-6), IL-1, tumor necrosis factor (TNF-α), monocyte chemotactic protein-1 (MCP-1), and C-reactive protein (CRP) (Yaribeygi et al. 2019). The C peptide is the result of posttranslational cleave product of proinsulin, whose fasting concentrations tendentiously rise (analogously with fasting insulin concentrations) in insulin-resistant states; being advocated, the

elevated levels of this biomarker are correlated with increased neoplastic burden risk and cancer-specific mortality (Yaribeygi et al. 2019). The higher levels of C peptide in patients with type 2 diabetes mellitus are associated with the recruitment of inflammatory cells to the subendothelial layer of the microvasculature, promoting a diffuse pattern of atherosclerosis and fostering the inflammatory microenvironment (Bhatt et al. 2014).

Overall, either by the senescence phenomena associated with aging or by obesity induced by diet, there is a tendentious modulation of adipose tissue immune microenvironment. Under physiological conditions and without excess of fat depots, there is an abundance of T regulatory cells responsible for the secretion of anti-inflammatory cytokines, as well as CD4+ T cells (T helper type 2), B regulatory cells, and macrophages that had undergone a polarization toward M2 phenotype. The M2 macrophagic polarization renders tolerogenecity and anti-inflammatory properties, a condition dependent upon peroxisome proliferator-activated receptor γ (PPAR-γ) stimulation and leading to the secretion of the anti-inflammatory IL-10. In situations of established overweight or obesity, there is a hyperplasia and hypertrophy of adipose tissue with a pro-inflammatory vascular stroma displaying an increase of macrophages that had undergone a polarization toward M1 phenotype (displaying a pro-inflammatory activity and whose differentiation is dependent on TNF-α and interferon-γ [IFN-γ]), neutrophils (that interact by CD11b with adipocytes intercellular adhesion molecule-1 [ICAM-1], ultimately leading to the production of ROS and formation of a web of extracellular fibers known as neutrophils extracellular traps formed by nucleic acids, proteins, and histones that will lead the activation of macrophages), natural killer cells, T CD8+ cells, and Th1 CD4+ cells. In senescence-associated microenvironmental changes, a shift toward M2 to M1 macrophage polarization is noted, as well as an increase in T CD8+ cells (Khan et al. 2020).

Adipose tissue per se is not an inert system, being considered an endocrine organ actively remodeled during metabolic reprogramming induced either by diet, senescence, or disease, in a continuous crosstalk with the immune system. Leptin is an orexinergic hormone secreted by white adipose tissue in response to their content in triglycerides, herein regulating energy stores with the intent to maintain body adiposity. At the central nervous system, leptin binds its receptors and promotes energy expenditure and negatively modulates the energetic balance (Pan and Myers 2018). Notwithstanding, leptin is also known to upregulate pro-inflammatory cytokines as IL-6 and TNF-α, rendering a basal pro-inflammatory state that serves as substrate for disease development (Lopez-Jaramillo et al. 2014). By contrast, adiponectin is secreted by adipocytes upon PPAR-γ stimulation and exerts anti-inflammatory effects, while increasing insulin sensitivity. Typically, its levels vary inversely to fat deposits. This adipokine acts by inducing the fatty acid oxidation, reducing gluconeogenesis, increasing glucose uptake, inhibiting oxidative stress, and decreasing monocyte to macrophage differentiation, with the expected decrease in TNF-α secretion (Lopez-Jaramillo et al. 2014).

Besides all of these players, active phase proteins synthesized by the liver as CRP and fibrinogen are also positively correlated with inflammation, being thoroughly used in clinical studies (Calder et al. 2013).

A brief description of the classical biomarkers and putative surrogate candidates (direct or indirect players in the physiological cascades abovementioned) is displayed in Table 1.

Nutrients and Inflammation

A considerable amount of literature has been published on the association between nutrients and inflammation. Overall, there is some evidence of the beneficial effect of n-3 PUFA (Calder 2017; Pan et al. 2022), vitamin C (Wannamethee et al. 2006; Oliveira et al. 2009; Jafarnejad et al. 2018), vitamin E (Oliveira et al. 2009; Garcia-Bailo et al. 2012; Asbaghi et al. 2020), magnesium (Kim et al. 2010; Dibaba et al. 2014), and flavonoids on inflammatory biomarkers.

Essential n-3 PUFA includes fatty acids such as DHA, EPA, and α-linolenic acid and must be obtained from diet. n-3 PUFA may modulate inflammation through a wide range of mechanisms, including modifying the profile of eicosanoids involved in inflammatory processes, affecting the production of inflammatory proteins and cytokines via alteration in gene expression in inflammatory cells and decreasing the expression of adhesion molecules while increasing pro-resolving lipid mediators produced from EPA and DHA (i.e., resolvins, protectins, and maresins) (Calder 2017). Khorsan et al. (2014) conducted a systematic review of randomized controlled trials (RCT) assessing the effect of n-3 PUFA interventions on inflammatory biomarkers among healthy and clinical populations. The authors observed an improved profile of inflammatory biomarkers with n-3 PUFA in critically ill patients and with cardiovascular disease; however, in healthy participants, n-3 PUFA had limited effects on inflammatory biomarkers. Likewise, a former review of RCT reported that n-3 PUFA supplementation overall had no effect on inflammatory biomarkers among healthy participants (Rangel-Huerta et al. 2012). In cancer patients, a meta-analysis of RCT by Pan et al. (2022) suggested that n-3 PUFA supplementation was associated with a reduction of serum CRP levels. Furthermore, in subgroup analyses a higher beneficial effect was seen for gastrointestinal and head and neck cancers as well as for the combination of EPA and DHA. By contrast, a meta-analysis of RCT found no association between CRP and TNF-α in diabetic or cardiovascular disease patients, respectively (Natto et al. 2019).

In contrast to the potential beneficial effect of n-3 PUFA on inflammation, saturated fatty acids (SFA) may have a pro-inflammatory effect, although evidence is not consistent (Minihane et al. 2015). A 3-week randomized crossover study in overweight/obese postmenopausal women found that replacing a diet rich in SFA (SFA, 29% of total energy intake; unsaturated fatty acids, 3% of total energy intake) to one rich in PUFA (PUFA, 25% of total energy intake; SFA, 8.5% of total energy intake) decreased serum CRP levels (Kralova Lesna et al. 2013). However, no significant change was observed for IL-18 concentrations. The LIPGENE Dietary Intervention Study was designed to determine the effect of replacing SFA with

Table 1 Classical biomarkers of inflammation and putative surrogate biomarkers

Class	Biomarker	Biological function
Enzymes	COX-1	Constitutively expressed and responsible for the conversion of AA to pro-inflammatory eicosanoids
	COX-2	Mostly inducible and responsible for the conversion of AA to pro-inflammatory eicosanoids
	Lipoxygenase 5	Responsible for the conversion of AA to LTB4
Fatty acids	Docosahexaenoic acid	Biomarkers of counter-inflammation (surrogate). Their relative abundance in the cell membrane of immune cells is associated with the synthesis of anti-inflammatory prostaglandins and leukotrienes
	Eicosapentahenoic acid	
Prostaglandins and leukotrienes	Prostaglandin-E2	Biomarker of inflammation. PGE2 is associated with vasodilation and increased vascular permeability. Results from AA conversion mediated by COX enzymes
	Leukotriene B4	Biomarker of inflammation. LTB4 is neutrophil chemoattractant. Results from AA conversion mediated by lipoxygenase enzymes
ROS products and related enzymatic catalyzers	Isoprostanes	Biomarkers or lipidic peroxidation. Their detection is dependent upon cathalization of AA from cell membranes in the presence of sources of free radicals in a reaction COX dependent
	Malondialdehyde	Biomarker of polyunsaturated fatty acids peroxidation
	Free nitrotyrosine (3-NO_2-Tyr)	Biomarker of protein oxidative nitration
	Myeloperoxidase	Indirect biomarker of oxidative stress; able to generate ROS that intervene in the innate immune response
Immunophenotypic hallmarks of inflammation in vascular stroma of adipose tissue	Neutrophils	Biomarker of inflammation associated with metabolic dysfunction. Increased in the vascular stroma of adipose tissue in overweight individuals
	Macrophages (M1 phenotype)	Biomarker of inflammation associated with metabolic dysfunction. Metabolic dysfunction and senescence are associated with a shift from M2 to M1 phenotype in macrophages of vascular stroma of adipose tissue
	NK cells	Biomarkers of inflammation associated with metabolic dysfunction
	T CD8+ cells	

(continued)

Table 1 (continued)

Class	Biomarker	Biological function
Cytokines	IL-1 beta	Neutrophil chemoattractant and activator. Activates B and T cells. Induces the synthesis of acute phase proteins by the liver. Binding its receptor in the hypothalamus induces fever
	IL-6	Induces neutrophil and lymphocyte activation and acute phase protein synthesis by the liver. Induces IL-10 secretion and inhibits TNF-α and IL-1
	MCP-1	Acts by binding its receptors (CCR1 and CCR2) expressed in monocytes and macrophages functioning as a leucocyte chemoattractant
	TNF-α	Leads to hepatic synthesis of acute phase proteins. Induces apoptosis and is correlated with cachexia development
	IFN-γ	Binds its receptors present in innate immune cells, NK cells, and T cells. Induces macrophagic and NK activation and upregulates MHC-I and MHC-II expression in antigen-presenting cells. Shift toward Th1 differentiation
Adhesion molecules	ICAM-1	Cell surface glycoprotein that leads to the adhesion of leukocytes to the endothelium. Expression is upregulated by pro-inflammatory cytokines. Also expressed in antigen-presenting cells, promoting T cell activation
	VCAM-1	Cell surface glycoprotein that leads to the adhesion of leukocytes to the endothelium. Expression is upregulated by pro-inflammatory cytokines and IL-4
Insulin-resistant states	C peptide	Biomarker of insulin-resistant states in type 2 diabetes, condition associated with systemic inflammation. Results from the cleave product of proinsulin and their fasting levels are tendentiously proportional to insulin resistance. Elevated levels are responsible for the immune cells' recruitment to subendothelial space leading to the development of atherosclerosis in a process highly dependent on inflammation
Adipokines	Leptin	Biomarker of inflammation and metabolic dysfunction. Orexinergic hormone secreted by adipose tissue in amounts proportional to their triglycerides content. Upregulates IL-6 and TNF-α

(continued)

Table 1 (continued)

Class	Biomarker	Biological function
	Adiponectin	Surrogate biomarker of inflammation. Physiologically exerts anti-inflammatory effects and increases insulin sensitivity; being reduced in conditions of metabolic dysfunction associated with inflammation
Endotoxins	LPS	Biomarker of inflammation and possibly metabolic dysfunction associated with dysbiosis. Present in the cell wall of gram-negative microbes
Liver acute phase proteins	C-reactive protein	Derivative of phosphatidylcholine residues. Involved in host defense by opsonization of pathogens and dead cells ultimately leading to complement activation
	Fibrinogen	Hepatic synthesis, soluble, being elevated in inflammatory conditions. Intervenient in the coagulation cascade, being converted to more insoluble fibrin in a reaction dependent on thrombin action

Physiological role of different mediators in the inflammatory response and their titration/measurement as an indicator of health status (Bhatt et al. 2014; Calder 2011; Fritsche 2015; Khan et al. 2020; Lopez-Jaramillo et al. 2014; Pan and Myers 2018; Yaribeygi et al. 2019)
AA arachidonic acid, *COX* cyclooxygenase, *COX-1* cyclooxygenase 1, *COX-2* cyclooxygenase 2, *ICAM* intercellular adhesion molecule, *IL* interleukin, *IFN-γ* interferon-γ, *LTB4* leukotriene B4, *LPS* lipopolysaccharide, *MCP-1* monocyte chemotactic protein-1, *MHC* major histocompatibility complex, *NK* natural killer, *PGE2* prostaglandin-E2, *ROS* reactive oxygen species, *Th* T helper, *TNF-α* tumor necrosis factor alpha, *VCAM* vascular cell adhesion molecule

monounsaturated fatty acids (MUFA) as part of an isoenergetic low-fat, high-complex carbohydrate diet in subjects with metabolic syndrome (Tierney et al. 2011). In that study, reduction of SFA had no effect on plasma CRP, IL-6, TNF-α, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), resistin, adiponectin, leptin, plasminogen activator inhibitor 1 (PAI-1), or tissue plasminogen activator (t-PA) concentrations.

The potential antioxidant properties are well known for vitamins C and E. Although there is some evidence of the anti-inflammatory effect of vitamins C and E, the results of observational and intervention studies are not consistent. In a cross-sectional study conducted in non-institutionalized adults, Oliveira et al. (2009) showed that vitamins C and E were inversely associated with CRP in men. However, cross-sectional data from the Multi-Ethnic Study of Atherosclerosis with adults aged 45–84 years and free of cardiovascular disease and diabetes showed that dietary intake of vitamins C and E was not significantly associated with IL-6, CRP, or fibrinogen (de Oliveira Otto et al. 2011). In a study conducted in 3258 men aged 60–79 years with no diagnosis of myocardial infarction, stroke, or diabetes, plasma vitamin C concentrations were inversely associated with CRP, fibrinogen, t-PA, and blood viscosity, while dietary intake of vitamin C was only associated with CRP and

t-PA (Wannamethee et al. 2006). Garcia-Bailo et al. (2012) examined the association between the serum concentrations of ascorbic acid, α-tocopherol, and 25-hydroxyvitamin D as biomarkers of vitamin C, E, and D nutritional status, respectively, and cytokines in young adults. This cross-sectional study found inverse associations between α-tocopherol concentrations and IFN-γ and regulated upon activation, normal T-cell expressed and secreted (RANTES), which, unlike ascorbic acid concentrations, were not associated with decreased inflammatory biomarkers. More recently, data from a cross-sectional study conducted with 10,808 individuals representative of the population of Spain aged ≥18 years found that dietary intake of vitamin E was not inversely associated with fibrinogen levels after adjusting for other nutrients (Padron-Monedero et al. 2021). Regarding the summary of current evidence, a meta-analysis of RCT revealed that the supplementation of vitamin E reduced serum levels of CRP around 0.52 mg/L, but overall, no significant effect on IL-6 and TNF-α was found (Asbaghi et al. 2020). Another meta-analysis conducted to examine the effects of vitamin C supplementation on CRP levels showed a significant effect of vitamin C in reducing circulating CRP concentrations, with stronger effect observed in subjects under 60 years, with CRP ≥3 mg/dL at baseline, at a lower dosage or intravenous administration (Jafarnejad et al. 2018). In addition, co-supplementation of vitamins C and E seems to have no significant effect on serum levels of CRP (Fouladvand et al. 2020).

Magnesium participates in several types of enzyme-mediated reactions that are essential for the proliferation, differentiation, and survival of cells. It has been described that magnesium might promote inflammation through priming phagocytes, improving oxidative bursts of granulocytes, increasing cytokine levels, and activating endothelial cells (Maier et al. 2021). In a 20-year follow-up prospective study with young American adults, inverse associations were found between magnesium intake and CRP, IL-6, and fibrinogen levels and between serum magnesium and CRP levels (Kim et al. 2010). The inverse association between serum CRP concentrations and magnesium intake was confirmed in a meta-analysis of seven cross-sectional studies (Dibaba et al. 2014). In contrast, a meta-analysis of RCT showed a significant reduction of CRP concentrations with magnesium supplementation only in individuals with inflammation, while the latter association was not significant in a whole analysis (Simental-Mendia et al. 2017). Similarly, a systematic review and meta-analysis of RCT in adult population reported that magnesium supplementation had no significant effect on CRP, IL-6, or TNF-α compared to the intervention control (Talebi et al. 2022).

Flavonoids are the most abundant polyphenols compounds in the human diet and are widely present in plant-based foods such as fruits, vegetables, legumes, tea, nuts, and seeds, among others. The anti-inflammatory effect of flavonoids encompasses several mechanisms, including inhibition of regulatory enzymes (protein kinases and phosphodiesterases) and enzymes participating in arachidonic acid metabolism (cyclooxygenase, phospholipase A2, and lipoxygenase), decrease of oxidative stress, and modulation of gene expression and immunological reprogramming (Maleki et al. 2019). Although in vitro studies and animal models have shown the protective effect of flavonoids against the inflammation cascade (Maleki et al. 2019),

studies in humans are still inconsistent. Two cross-sectional studies with data from the National Health and Nutrition Examination Survey 1999–2002 found conflicting results. The first study found that flavonoid intake (as quartiles of consumption) was inversely associated with CRP concentrations (Chun et al. 2008); in contrast, the second one failed to find a significant association between flavonoid intake (as quintiles of consumption) and CRP concentrations (Floegel et al. 2011). Nonetheless, other cross-sectional studies have revealed significant associations between flavonoid consumption and inflammatory biomarkers (Jennings et al. 2014; Vernarelli and Lambert 2017; Hsieh et al. 2021).

There is a growing body of literature that recognizes the potential effect of dietary sugar intake on increasing the basal pro-inflammatory state. Apart from other potential mechanisms, the promotion of the inflammation seems to be triggered by the production of free fatty acid metabolites derived from the glucose-fatty acid cycle that is promoted by dietary sugar consumption (Alkhouri et al. 2009; Softic et al. 2016; Della Corte et al. 2018). A population-based cross-sectional study of 9678 adults found that a higher intake of sugars from liquids was associated with CRP, while no association was found for sugars from solid food (O'Connor et al. 2018). Another cross-sectional study of adults found a significant association between naturally occurring sugars from foods and CRP; however, this association was no longer significant when the analysis considered sociodemographic and lifestyle variables and diet quality (Bergeron et al. 2021). In a prospective study of data from the DOrtmund Nutritional and Anthropometric Longitudinally Designed Study, dietary intake of glucose; fructose; sucrose; total sugar; free sugar; added sugar; total sugar from sugar-sweetened beverages, juice, and sweets; and urinary sugars (i.e., fructose and sucrose) during adolescence (girls, 9–15 years; boys, 10–16) were not associated with pro-inflammatory scores (based on CRP, IL-6, IL-18, leptin, chemerin, adiponectin) in adulthood (18–36 years), with the exception of sugar from sugar-sweetened beverages, which was associated with an increased pro-inflammatory score only among females (Della Corte et al. 2020). Regarding the differential effect of dietary sugars, results from a meta-analysis of 13 intervention studies, consisting of 1141 individuals aged $\geq$11 years, did not support the contribution of fructose for subclinical inflammation being higher than dietary glucose or sucrose (Della Corte et al. 2018).

Food and Inflammation

The role of food intake (as individual food or food group) in relation to inflammatory processes has been the subject of many studies in the last years.

Fruits and vegetables have raised particular interest due to their content in vitamins, fiber, antioxidants, and polyphenolic compounds. Oliveira et al. (2009) reported that higher consumption of fruits and vegetables are cross-sectionally associated with lower CRP levels only in men, with an attenuated effect in overweight participants. Likewise, in a cross-sectional study with 4027 adults from the CoLaus study, a negative association was found between fruit intake

and CRP levels but not with other inflammatory biomarkers (Piccand et al. 2019). In addition, there is no significant association between vegetable intake and the levels of CRP, IL-6, and TNF-α. Other cross-sectional studies have observed a similar protective effect of fruit and vegetable intake on inflammatory biomarkers in adult (Esmaillzadeh et al. 2006; Wannamethee et al. 2006; Jiang et al. 2014) and pediatric populations (Holt et al. 2009; Almeida-de-Souza et al. 2018). Accordingly, a meta-analysis of intervention studies of adults showed that a higher intake of fruits and vegetables was associated with lower levels of CRP and TNF-α but not with IL-6 concentrations (Hosseini et al. 2018). On the other hand, interestingly, Schwedhelm et al. (2019) examined whether the consumption of foods such as fruits and vegetables at different meals (breakfast, lunch, and dinner) may influence an association with cardiometabolic status and inflammatory biomarkers. Findings of that study showed that fruit intake at dinner was inversely associated with CRP, but no association was found for vegetable intake at different meals. However, vegetable intake at breakfast was inversely associated with low-density lipoprotein cholesterol, while vegetable intake at dinner was positively associated with high-density lipoprotein cholesterol. In addition to the importance of the quantity of food consumed, the timing of food intake may be considered as a probable factor affecting metabolic milieu.

Over the past decades, there has been a dramatic increase in the evidence on the role of dairy product intake on inflammatory profile. Indeed, some dairy components, such as unsaturated fatty acids, proteins, amino acids, calcium, and magnesium, may contribute to a decreased risk of inflammation (Da Silva and Rudkowska 2015). A systematic review from RCT conducted to assess the impact of dairy consumption on biomarkers of inflammation among overweight and obese adults suggested that dairy products had no adverse effect on the biomarkers of inflammation (Labonte et al. 2013). In that review, the authors stated that, due to the methodological factors and limitations of the eight included studies, no conclusion could be drawn of the beneficial or neutral effect of dairy consumption on inflammatory biomarkers. Likewise, Ulven et al. (2019), due to the low quality of the studies included in the review, did not identify an anti-inflammatory effect of milk or dairy intake, only suggesting that their consumption did not show a pro-inflammatory effect in healthy subjects or in subjects with an acute or chronic disease (i.e., overweight, obese, metabolic syndrome, or type 2 diabetic patients). In contrast, more recently, another systematic review with a meta-analysis of RCT highlights the possible anti-inflammatory effect of dairy intake (Moosavian et al. 2020). In that meta-analysis, high dairy product consumption showed a decrease in serum levels of CRP, TNF-α, IL-6, and MCP-1 and increased levels of adiponectin compared to low or no dairy product intake. However, when subgroup analysis was done for study design, no significant associations were found in crossover studies. In addition, there is no significant association between dairy product intake and the serum level of leptin, ICAM-1, or VCAM-1. Although the majority of the studies on the association of dairy products and inflammation do not consider the type of dairy product, it is important to highlight that the unique food matrix of each dairy product might lead to different effects on inflammatory biomarkers.

The relationship between other food or food groups and inflammation has been systematically reviewed. Olive oil is a key component of the Mediterranean diet (MedD) and has been overall associated with a lower risk of cardiovascular disease and inflammation (Schwingshackl et al. 2015). The high content in MUFA and phenolic compounds in olive oil may explain its antioxidant activity and anti-inflammatory properties (Lucas et al. 2011; Schwingshackl and Hoffmann 2014b). In this line, a systematic review and meta-analysis of RCT on the effect of the dietary intake of olive oil on inflammatory biomarkers found that regular consumption of olive oil decreased IL-6 compared to controls, with a stronger association when compared to a low-fat diet (Fernandes et al. 2020). For CRP, no significant effect was observed.

Some studies have investigated the effect of seed and nut consumption on markers of inflammation. Nuts and seeds contain a variety of nutrients and bioactive compounds, such as α-linolenic acid, fiber, and vitamin E, that might protect against inflammation (Vinson and Cai 2012; Khalatbari Soltani et al. 2013). A meta-analysis of 23 RCT showed that, overall, nut consumption significantly decreased ICAM-1 levels. However, in subgroup analysis, significant results remain only for parallel or long-term ($\geq$12 weeks) studies. There is no significant effect of nut consumption for CRP, VCAM-1, IL-6, TNF-α, or E-selectin. A previous systematic review and meta-analysis revealed a favorable effect of nut consumption on endothelial function but not in levels of CRP, adiponectin, TNF-α, IL-6, ICAM-1, or VCAM-1 (Neale et al. 2017). In turn, a meta-analysis of the effect of flaxseed supplementation with 2520 adults found that flaxseed supplements reduced the levels of CRP, IL-6, and VCAM-1, but not for TNF-α, ICAM-1, or E-selectin concentrations (Askarpour et al. 2020). On the other hand, a former meta-analysis of 17 RCT with 1256 individuals suggested that flaxseed supplementation had no effect on CRP levels (Ursoniu et al. 2019).

As with other foods, red meat's role in causing inflammation has been investigated. Although some observational studies have reported a negative impact of red meat consumption as an inflammatory biomarker (Chai et al. 2017; Schwedhelm et al. 2017), a recent meta-analysis of RCT with a short duration (3–16 weeks) found no association between red meat intake and fasting glucose, insulin, CRP, IL-6, or TNF-α (O'Connor et al. 2021). However, the objects focused on in the included studies consisted mainly of unprocessed red meat.

Dietary Patterns and Inflammation

Although the literature highlights the role of nutrients in the inflammation process, the study of the effect of individual nutrients or foods does not account for the interactions between nutrients or the complexity of the combination of nutrients that occur in diet (Newby 2007). Hence, in recent decades, the study of dietary patterns has been widely used in nutritional epidemiology, as it provides a closer picture of the whole diet.

The study of the association between dietary patterns and biomarkers of inflammation has been based in the a priori approach using diet scores or indices (e.g., the Mediterranean score, the Healthy Eating Index, or the Dietary Inflammatory Index [DII]) or the a posteriori data-driven approach identifying dietary patterns through principal component analysis, factor analysis, or cluster analysis (Barbaresko et al. 2013; Hart et al. 2021). A systematic review including 43 cross-sectional and 3 experimental studies investigated the effect of dietary patterns derived by a priori and data-driven approaches on inflammatory biomarkers (Barbaresko et al. 2013). A posteriori dietary patterns as western-type and meat-based diet were positively associated with inflammatory biomarkers, while vegetable- and fruit-based patterns were inversely associated with inflammatory biomarkers, predominantly CRP, IL-6, and fibrinogen. Of the 46 included studies, 21 analyzed dietary scores or indices, mostly MedD and Healthy Eating Index. The majority of the latter studies reported positive associations with adiponectin and IL-10, and negative associations with CRP, IL-6, IL-8, and fibrinogen. The three experimental studies that applied MedD supported an inverse association with inflammatory biomarkers. In a meta-analysis of RCT, MedD was likewise associated with a reduction of inflammatory biomarkers (Schwingshackl and Hoffmann 2014a). Compared to control diets, the Mediterranean pattern significantly increased adiponectin and decreased CRP and IL-6. The PREDIMED (PREvención con DIeta MEDiterránea) clinical trial with elderly adults at a high cardiovascular risk showed that MedD interventions (MedD supplemented with extra-virgin olive oil or MedD supplemented with nuts) decreased IL-6, CRP, TNF-α, and MCP-1 in the short and long term (Urpi-Sarda et al. 2012; Casas et al. 2016). In addition, findings from a sub-study of PREDIMED trial reported that MedD interventions reduced the risk of developing mild and severe leukopenia or leukocytosis in geriatric individuals without abnormal cell counts at enrolment and that a high adherence to MedD decreased the incidence of disrupted white blood cell counts as well as an all-cause mortality risk related to leukopenia (Hernaez et al. 2021). The MedD is one of the most widely studied dietary patterns in the context of inflammation and other health outcomes; it is characterized by a high consumption of legumes, fruits, nuts, whole grains, and olive oil as the main source of added fat; a moderate intake of fish, dairy products, eggs, and wine (mostly red wine); and a low intake of meat, particularly red meat, and processed foods (Trichopoulou and Vasilopoulou 2016). It has been proposed that the beneficial role of the MedD, due to its content in antioxidants, MUFA, PUFA, and short-chain fatty acids, may be overall explained by a neutralization of ROS, an improvement of autophagy and immune cell balance, and a downregulation of the expression of cell adhesion molecules and endothelial dysfunction (Tsigalou et al. 2020).

Other dietary patterns may also reduce inflammatory biomarkers such as the Dietary Approaches to Stop Hypertension (DASH) diet, DII, vegan, and vegetarian diets. A meta-analysis by Soltani et al. (2018) showed that the DASH diet reduced CRP serum levels compared to habitual diets; however, when compared to healthy diets, the effect was not significant. Additionally, when analysis was stratified for study duration, the reduction in CRP levels was only significant for trials lasting 8 weeks or more. A systematic review of the evidence on the associations between

dietary patterns and inflammatory biomarkers in the adult population showed in four of the six analyses included that a higher adherence to the DASH diet was associated with lower levels of inflammatory biomarkers (Hart et al. 2021). Furthermore, the DASH diet was positively associated with adiponectin.

The association of DII and inflammatory biomarkers was first reported by Cavicchia et al.'s longitudinal observational study (2009), which showed an inverse association between DII scores and CRP. Likewise, other studies with cross-sectional (Phillips et al. 2018; Shin et al. 2019) and longitudinal design (Shivappa et al. 2014) have reported a positive association between DII and inflammatory biomarkers among adults. In a cross-sectional study with 1992 adults, Phillips et al. (2018) found that participants with higher DII energy-adjusted scores had higher complement component 3, CRP, IL-6, TNF-α concentrations, higher white blood cell count and neutrophil-to-lymphocyte ratio, and lower adiponectin levels compared to their counterparts. In addition, DII was associated with an unfavorable lipoprotein profile and increased metabolic syndrome risk. On the other hand, Ren et al. (2018) showed that higher DII scores were associated with higher CRP among subjects with metabolic syndrome but not in the whole sample, highlighting that the effect of DII may differ according to health status. The overall beneficial effect of DII is not restricted to the adult population. In children and adolescents, a systematic review on the association between the DII and cardiometabolic risk and inflammatory biomarkers showed that a more pro-inflammatory diet is associated with higher levels of IL-1, IL-2, and IL-6, TNF-α, IFN-γ, and VCAM-1 (Suhett et al. 2021). On the other hand, none of the included studies identified a significant association between DII and CRP. Nonetheless, these results must be interpreted with caution since only two of the six included observational studies had examined the association between DII and inflammatory biomarkers.

Plant-based diets, such as vegan or vegetarian diet, have had a growing adherence in recent years in Western countries (Menzel et al. 2020). A recent meta-analysis of cross-sectional studies found that vegans had lower levels of CRP compared to omnivore subjects. For vegetarians, the latter association was weaker in apparently healthy individuals, while in patients with impaired kidney function, the association between vegetarian diet and CRP was stronger (Menzel et al. 2020). In addition, no significant associations were seen for IL-6, IL-18, IL-1 receptor antagonist, TNF-α, E-selectin, ICAM-1, MCP-1, adiponectin, omentin-1, or resistin. However, a former meta-analysis of 16 cross-sectional studies showed that vegetarian diets compared to omnivorous diets were only associated with lower serum levels of CRP and higher levels of IL-6 for those who followed the diet for at least 2 years (Haghighatdoost et al. 2017). Craddock et al. (2019) also conducted a systematic review and meta-analysis of observational and intervention studies to evaluate the relationship between vegetarian-based dietary patterns and inflammatory and immune markers. The meta-analysis was only computed for observational studies due to insufficient data for intervention studies. The authors found that vegetarian-based diets were associated with lower concentrations of CRP and fibrinogen compared to their non-vegetarian-based counterparts.

Conclusion

The evidence reviewed suggest that a variety of components of isolated or combined dietary components can modulate inflammation. However, findings still conflict or are inconsistent with high heterogeneity across studies. The discrepancies in the findings of existing studies may be due to differences in design, intervention time, inflammatory biomarkers, statistical methodologies, health status of participants, and inadequate control of potential confounders. Most investigations on the association between diet and inflammation have been cross-sectional, with longitudinal studies leaning toward no or weaker associations compared to cross-sectional studies (Hart et al. 2021). Regarding dietary patterns, the existing methodology used to compute them is widely heterogenous in both a priori and a posteriori approaches, with discrepancies ranging from the methods used to evaluate dietary intake differences between studies to the simple prompt availability of food analysis composition. Dietary patterns derived from a single day, for example, do not account for day-to-day variation of a diet, which can lead to misclassification and attenuate the association between dietary patterns and inflammation (Tucker 2010; Barbaresko et al. 2013). In addition, some computed scores or indices of the existing studies did not include all nutritional/food items originally defined, leading to a different combination of nutrients/foods and a sub-representation of the healthy potential of a particular dietary pattern. On the other hand, a posteriori approaches explore the existing dietary patterns in a specific population, so the dietary patterns derived are study-population- and data-specific, hindering the comparison between studies (Tucker 2010; Ocke 2013). Furthermore, the beneficial effect of diet on some inflammatory biomarkers may be stronger in populations with a higher risk of inflammation such as individuals with obesity, old age, nutritional deficiency, or other chronic disease (Padron-Monedero et al. 2021). In addition, the analysis of inflammatory biomarker must be cautious and based on panels of multiple conventional biomarkers instead of relying on a single one, given their not infrequent context-dependent role and titration singularities.

Despite the limitations of the research on the role of diet in inflammation with inherent immunological reprogramming, diet is a key target in prevention and treatment of numerous chronic diseases triggered by slow damage accumulation highly promoted by a chronic pro-inflammatory state.

Applications to Prognosis and Other Diseases or Conditions

Applications to Prognosis

Not applicable

Applications to Other Diseases or Conditions

In this chapter we reviewed evidence on the association between diet and inflammatory biomarkers in humans. Although most of the available evidence suggested a

beneficial effect of n-3 PUFA, vitamins C and E, magnesium, flavonoids, fruits and vegetables, plant-based dietary patterns, MedD, and anti-inflammatory diets, findings are still inconsistent. In addition, this effect may be stronger in non-healthy population.

Key Facts of Cytokines

- CRP, IL-6, and TNF-α are the most used inflammatory biomarkers in nutritional studies among humans.
- Pro-inflammatory diets usually lead to an increase in the abovementioned biomarkers.
- Dosing of IL-6 has some drawbacks: its determination is not available in all clinical settings; sequential measurements are required; its production can be inhibited by immunosuppressive drugs as steroids, and it may be influenced by chronic kidney disease and renal replacement therapy.
- CRP is mainly present in two isoforms: pentameric CRP (pro-inflammatory or anti-inflammatory, depending on the context) and monomeric CRP (pro-inflammatory), being activated platelets important for the conversion of pentameric isoform into monomers.
- Analysis of CRP levels must be aware of confounding factors such as the presence of infection, neoplasia, or liver injury that may elevate their concentrations.

Summary Points

- The inflammatory response, when meeting the homeostatic and physiological demands, has a proven benefit (e.g., by eliminating pathogens).
- The disruption of the signaling cascades (that are interconnected) in the inflammasome may endeavor a perpetuation of a pro-inflammatory status that is associated with disease development.
- Senescence by itself is associated with a pro-inflammatory status, being modulated by environmental expositions (as diet).
- CRP, IL-6, and TNF-α are the most used inflammatory biomarkers in nutritional studies among humans.
- Most of the studies on the role of diet in inflammation are cross-sectional.
- Diet may modulate inflammation through a wide range of mechanisms.
- Components of diet such as vitamins C and E, magnesium, n-3 polyunsaturated fatty acids, flavonoids, fruits, vegetables, and plant-based dietary patterns may have a beneficial effect on inflammation.

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YKL-40 as an Inflammatory Biomarker in Nutrition

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Abstract

Low-grade inflammation plays an important role in several development processes of diseases. Chitinase-3-like protein 1 (YKL-40) is a glycoprotein, secreted by macrophages, neutrophils, and different cell types, and it is also associated with inflammation and pathological tissue remodeling in diseases. YKL-40 are the new potential biomarkers of inflammation. Here, we review an important role of YKL-40 through cytokines and signaling pathways as a candidate inflammatory biomarker of nutrition.

Keywords

YKL-40 · Nutrition · Inflammation · Signal pathways · Cytokines

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Abbreviations

1,25(OH)2D3	1α,25-Dihydroxyvitamin D3
AD	Alzheimer's disease
ALA	a-Linolenic acid
ALI	Acute lung injury
AMI	Acute myocardial infarction
ASK1	Apoptosis signal-regulator kinase
BMI	Body mass index
C/EBP	CCAAT/enhancer-binding protein
CAD	Coronary artery disease
CHI3L1	Chitinase-3-like protein 1
CM	Curcuma longa
COMP	Cartilage oligomeric matrix protein
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
ERKs	Extracellular signal-regulated protein kinases
ESR	Erythrocyte sedimentation rate
FA	Food allergy
HOMA-IR	Homeostasis model assessment of insulin resistance
hsCRP	High-sensitivity C-reactive protein
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor-I
IL	Interleukin
IR	Insulin resistance
JAK-STAT	Janus family-signal transducers and activators of transcription
JNK	c-Jun N-terminal kinases
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein
ME	*Morchella esculenta*
MMP	Matrix metalloproteinase
MO	Morbid obesity
MP	Milk-based protein
MS	Metabolic syndrome
NAFLD	Nonalcoholic fatty liver disease
NF-kB p65	Nuclear factor-kappa B p65
NF-kB	Nuclear factor-kappa B
Nrf-2	Nuclear factor-erythroid 2-associated factor 2
OA	Osteoarthritis
OSM	Oncostatin M
PBMC	Peripheral blood mononuclear cells
PCI	Percutaneous coronary intervention
PI3K	Phosphatidylinositol 3-kinase
RYGB	Roux-en-Y gastric bypass

SAPK	Stress-activated protein kinase
SCAAT	Subcutaneous abdominal adipose tissue
SP	Soy protein
T2DM	Type 2 diabetes mellitus
TGF beta	Transforming growth factor beta
Th2	T helper 2
TNF-α	Tumor necrosis factor-α
VAT	Visceral adipose tissue
VDR	Vitamin D receptor
WHR	Waist-hip ratio

Introduction

Nutrition has been shown to have effects on metabolism by the expression of key regulatory signaling proteins via cytokines and hormones (Alwarawrah et al. 2018). Changes in nutritional status affect the organ size, hormone and cytokine levels, and immune cell population and function. In mammals, there are many processes to control systemic nutrient use and storage. For example, nutrients taken in excess of the body's needs are stored in adipose tissue, liver and muscle. Then, the stored nutrients are metabolized to provide energy and building blocks to sustain vital physiological processes (Alwarawrah et al. 2018). A change in adipose tissue volume in response to under- or overnutrition causes the secretion of hormones and cytokines. Interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α are pro-inflammatory cytokines secreted from adipose tissue in response to tissue damage and obesity. IL-6 also induces the production of pro-inflammatory cytokine IL-17 and interferon gamma (IFN-γ) by promoting T cell differentiation. Macrophages polarize into activated macrophages (formerly called M1 macrophages) in response to IFN-γ secreted in adipose tissue. This inflammatory macrophages lead to increased secretion of cytokines such as TNF-α, monocyte chemotactic protein (MCP)-1, IL-1β, IL-6, and IL-12. In lean individuals, alternatively activated macrophages (M2 macrophages) secrete anti-inflammatory cytokines like adiponectin, IL-10, and IL-4. Leptin secreted by adipocytes increases in obesity and decreases in malnutrition (Alwarawrah et al. 2018). The functioning of the pathways involved in the perception and management of nutrients ensures the regulation of metabolic homeostasis and is therefore important for survival (Hotamisligil and Erbay 2008). Chronic nutrient deficiency or pervasive energy excess disrupts the delicate balance between immune and metabolic responses and causes some diseases. A balanced diet and adequate energy flow are necessary for the proper functioning of the immune system and metabolic pathways. In addition to cytokines, increased circulating lipids as a result of nutritional disorders trigger inflammatory signaling pathways that inhibit insulin receptor signaling (Hotamisligil and Erbay 2008).

There are some searches of the key signaling pathways with regard to metabolism inflammation and insulin action. As shown by Hotamisligil and Erbay (2008), obesity induces the activation of c-Jun N-terminal kinases (JNKs) in metabolically active

organs like the liver, muscle, and adipose tissue. Various stress signals such as pro-inflammatory cytokines, free fatty acids, reactive oxygen species, pathogens, and pathogen-associated components in obesity lead to inhibition of insulin signaling by JNK activation (Hotamisligil and Erbay 2008). Chronic low-grade inflammation in adipose tissue triggers systemic inflammation and metabolic disorders resulting in insulin resistance (IR), the development of type 2 diabetes mellitus (T2DM), and cardiovascular disease (CVD) (Kim et al. 2016). The adipocyte in lipid-laden adipose tissue in obesity produces MCP-1 facilitating the recruitment of circulating monocytes and infiltration of macrophages (Kim et al. 2016). The mitogen-activated protein kinase (MAPK) family consists of three main members: extracellular signal-regulated protein kinases (ERKs), JNKs, and p38 kinases (Zhang et al. 2016). Inflammatory cytokines and many other cellular stresses may activate the p38 MAPK pathway taking part in apoptosis and cell cycle regulation (Zhang et al. 2016). As described previously (Fernández-Riejos et al. 2008), leptin activates receptor-associated kinases of the Janus family-signal transducers and activators of transcription (JAK-STAT), MAPK, and phosphatidylinositol 3-kinase (PI3K) signaling pathways in human peripheral blood mononuclear cells (PBMC) (Fernández-Riejos et al. 2008). Leptin stimulating MAPK and MEK-1/2 pathways leads to ERK1/2 transducing cellular growth and proliferation. Also, in Jurkat T cells, it regulates insulin sensitivity via PI3K signaling in hypothalamic neurons. ERK phosphorylation is a significant process of activation of the RAF/MEK/ERK signaling pathway (Fuentes et al. 2011). AKT phosphorylation is an important condition of the activation of the PI3K/AKT pathway regulating cell survival and growth. The activation of the MAPK/ERK and PI3K pathways is regulated by nutritional status (Fuentes et al. 2011).

Obesity is a pro-inflammatory condition that causes adipocytes to enlarge due to excessive fat deposition resulting in the secretion of certain molecules and cytokines (Jayarathne et al. 2018). More than 50 hormones and signaling molecules secreted by adipose tissue, collectively called adipokines, have autocrine, paracrine, and systemic biological roles and affect numerous physiological processes related to energy, glucose metabolism, and immunity. Adipokines secreted from adipose tissue are either anti-inflammatory or pro-inflammatory. Secretion of inflammatory adipokines increases macrophage infiltration into adipose tissue. Therefore, increased circulating levels of pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β in obesity contribute to the development of IR. Leptin is well known to regulate food intake and body weight; but it also exhibits pro-inflammatory activities by upregulating pro-inflammatory cytokines, such as TNF-α, IL-12, and IL-6. Nuclear factor-kappa B (NF-kB) is a major inflammatory transcription factor which regulates many downstream genes associated with inflammation such as IL-6, MCP-1, and TNF-α (Jayarathne et al. 2018).

General Characteristics of YKL-40

YKL-40 is a 40-kDa heparin- and chitin-binding glycoprotein also known as human cartilage glycoprotein 39 (HCgp39) or chitinase-3-like protein 1 (CHI3L1) (Rathcke and Vestergaard 2009). The CHI3L1 gene for human YKL-40 is localized on

chromosome 1q31-q32. YKL-40 is a member of the 18 glycosyl hydrolase family containing chitinase, but YKL-40 doesn't have glycohydrolase activity. YKL-40 secreted in a variety of cells such as activated macrophages, neutrophils, arthritic chondrocytes, fibroblast, synovial cells, osteoblasts, and differentiated vascular smooth muscle cells causes activation of the innate immune system and some cell processes leading to extracellular matrix remodeling (Rathcke and Vestergaard 2009). High circulating serum YKL-40 levels have been found in many inflammatory and tissue remodeling conditions such as cancer, osteoarthritis (OA), rheumatoid arthritis, liver fibrosis in nonalcoholic fatty liver disease (NAFLD), IR, obesity, endothelial dysfunction, atherosclerosis, and CVD (Kwon et al. 2020). YKL-40 is related to migration, reorganization, and adhesion of endothelial cells and smooth muscle cells in angiogenesis (Schernthaner et al. 2016). YKL-40 associated with metabolic syndrome (MS) (Akboğa et al. 2016), morbid obesity (MO) (Hempen et al. 2009), T2DM (Brix et al. 2011), T1DM (Rathcke et al. 2009), and albuminuria (Brix et al. 2011) suggests an interaction in the development and progression of atherosclerosis in patients with those comorbidities. The biological function of YKL-40 in nutritional processes has not yet been determined.

The Effects of YKL-40 on Signaling Pathways Related to Nutrition and Metabolism

There are different studies on the effect of YKL-40 on signaling pathways related to nutrition and metabolism. YKL-40 interacts with different signaling pathways.

As described previously (Mansell et al. 2016), 1α,25-dihydroxyvitamin D3 (1,25 (OH)2D3) and a rich source of the pleiotropic lipid mediator, lysophosphatidic acid (LPA), synergistically induced YKL-40 expression in the human osteosarcoma-derived osteoblast, MG63 cells. The AP1, MEK, Sp1, and STAT-3 inhibitors suppressed the expression of both alkaline phosphatase (ALP) and YKL-40 by MG63 cells in response to co-stimulation with 1,25(OH)2D3 and LPA. It was thought that increased YKL-40 by LPA (MEK activation) and 1,25(OH)2D3 could be via MEK-linked stimulation of AP-1. In MG63 cells co-treated with 1,25(OH) 2D3 and LPA, UO126, an inhibitor of MEK, can suppress the production of YKL-40. Also, YKL-40 expression could be suppressed using mithramycin A inhibiting Sp1 promoter binding. The protein kinase C activator, phorbol 12-myristate 13-acetate, and 1,25(OH)2D3 synergistically elevated YKL-40 production in MG63 cells. It was thought that MG63 maturation was induced by the high expression of ALP and YKL-40. Also, suppressing AP-1 and STAT3 (essential in mediating cell proliferation, differentiation, and survival) with SR11301 and S31-201 respectively inhibited YKL-40 expression in osteoblasts co-stimulated with LPA and 1,25(OH)2D3. A major polyphenol of turmeric (curcumin longa, (CM)) without LPA increased YKL-40 production. Like CM, the purple fruit-derived anthocyanidin Del failed to cooperate with LPA to induce both ALP and YKL-40 production by MG63 cells (Mansell et al. 2016). As described previously (Rehli et al. 2003), YKL-40 expression connecting with an Sp1 element increased in

phorbol ester-mediated differentiation of human macrophage cell line (THP-1). The vitamin D receptor (VDR) may increase the stability of Sp1-GC-rich DNA interaction and stimulation of target genes such as YKL-40 and ALP by an Sp1 transactivation domain. The study previously described for YKL-40 protein expression by immunohistochemistry consisted of sections from 15 human embryos (weeks 5.5–8) and 68 fetuses (weeks 9–14) (Johansen et al. 2007). Initially YKL-40 mRNA expression and YKL-40 protein expression were found in tissues of the ecto-, meso-, and endoderm and during the development of the cartilage, bones, joints, and muscles. At the cellular level, YKL-40 protein expression is high in tissues characterized by rapid proliferation, marked differentiation, and undergoing morphogenetic changes during the development of the human musculoskeletal system (Johansen et al. 2007). YKL-40 initiated MAPK and PI3K signaling cascades leading to phosphorylation of ERK1/2 and AKT in fibroblasts resulting in mitogenesis and cell survival. YKL-40 mRNA expression and protein synthesis by human articular chondrocytes, rat chondrocytes, and osteoblasts are stimulated by TNF-α- and IL-1-linked NF-kB activity (Recklies et al. 2005). YKL-40 regulation of p38 and SAPK/JNK is mediated by PI3K, and secretion of YKL-40 required sustained activation of NF-kB (Rathcke and Vestergaard 2009). YKL-40 binds specifically to collagen types I, II, and III and regulates the type I collagen fibril formation (Bigg et al. 2006). Furthermore, YKL-40 contributes to chondrocyte differentiation by enhancing the transcription factor SOX9 and type II collagen expression. The induction of SOX9 depends on ERK1/2 and PI3K activities but not on p38 and JNK/MAPK (Jacques et al. 2007). The YKL-40 promoter sequence also includes consensus binding sites for several known transcription factors such as nuclear PU.1, Sp1, Sp3, upstream stimulatory factor, acute myeloid leukemia, and CCAAT/enhancer-binding protein (C/EBP) (21). The expression of YKL-40 is controlled via some cytokines and hormones, such as TNF-α, IL-1ß, IL-6, IL-13, IFN-ɣ, vasopressin, and parathyroid hormone-related protein (Lee et al. 2011).

In a study, the authors showed that IL-1 together with IL-6 or oncostatin M (OSM) synergistically increased YKL-40 expression in both primary human and mouse astrocytes in vitro (Bhardwaj et al. 2015). The YKL-40 in astrocytes was increased by both active STAT3 and RelB and p50 subunits of NF-κB and suppressed through dominant-negative IκBα. IL-1 promoted by RelB/p50 complex formation was further stimulated by OSM. Next, these complexes directly were bound to the YKL-40 promoter. YKL-40 triggers the interaction of vß3 integrins with syndecan-1 in endothelial cells and activates ERK, AKT, and Wnt/ß-catenin signaling in macrophages by IL-13 receptor alpha 2-dependent mechanism (Bhardwaj et al. 2015). It was declared that YKL-40 activated the PI3K/AKT cascade to increase IL-18 production in osteoblasts and diminish the levels of miR-590-3p. Here, both the pharmacologic inhibitors and siRNAs of PI3K and AKT suppressed IL-18 production and EPC angiogenesis. Transfection of osteoblasts with miR-590-3p mimic decreased IL-18 expression and EPC angiogenesis. miR-590-3p is therefore a negative regulator in YKL-40-induced EPC tube formation and migration (Li et al. 2017). YKL-40, a growth factor for fibroblasts, chondrocytes, and synovial cells, acted synergistically with insulin-like growth factor-I (IGF-I) and was mediated via TNF-α and IL-6 (Rathcke and Vestergaard 2009).

The Role of YKL-40 in Obesity and Diabetes

There are different studies on the relationship between IR, obesity, diabetes, and YKL-40 level. YKL-40 is closely related to obesity and MS that resulted from nutritional excess. In a study, high levels of YKL-40 in MO patients decreased after massive weight loss followed by bariatric surgery. After a mean follow-up of 17.4 months and a mean weight loss of 40 kg after bariatric surgery, YKL-40 levels decreased by 30.5%. YKL-40 was correlated with homeostasis model assessment of insulin resistance (HOMA-IR) and fasting insulin levels, and this indicated a role in the developing processes of IR and T2DM. The tight association of MCP-1 (plaque development) and YKL-40 (plaque rupture) contributes to the increased cardiovascular mortality in MO patients (Hempen et al. 2009).

In another study, the authors showed that the serum levels of both YKL-40 and hs-CRP were significantly raised in patients with MS (Akboğa et al. 2016). As shown by Thomsen et al. (2013), the inflammatory markers YKL-40 and MCP-1 were elevated in MO patients and declined after weight loss. Fasting MCP-1 levels reduced after Roux-en-Y gastric bypass (RYGB) in subjects with T2DM and normal glucose tolerance, whereas fasting YKL-40 levels were unchanged. YKL-40 levels showed a slight postprandial suppression on all study days in the T2DM group. As described previously (Thomsen et al. 2015), fasting serum YKL-40 levels were positively related with measures of obesity and dyslipidemia including waist-hip ratio (WHR) and fasting plasma triglyceride (TG) levels. Visceral adipose tissue (VAT) could be the main source of YKL-40 which could be related to low-grade inflammation in obesity. There wasn't any association between YKL-40 and IR or insulin sensitivity. This indicated that YKL- 40 was not directly involved in the key pathophysiological features of T2DM (Thomsen et al. 2015). In the obesity study, PBMC gene expression of the YKL-40 was upregulated in obesity, independent of the glycemic state, and related to different circulating inflammatory markers and IR with impaired extracellular matrix remodeling. Also, elevated YKL-40 gene expression in PBMC was found to cause the inflammatory response to worsen in obesity. Interestingly, a positive correlation with YKL-40 and circulating markers of liver function such as AST and ALT was defined (Catalán et al. 2015). The authors showed that serum YKL-40 levels were significantly higher in the high-density lipoprotein (HDL-C) and high-TG groups but were not correlated with total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels. It is now known that the plasma YKL-40 levels were also higher in the high atherogenic index of children group. According to these findings, it was offered that serum YKL-40 may be a useful initial screening tool or follow-up risk indicator for lipid abnormalities, atherosclerosis, and CVD in children and adolescents (Kwon et al. 2020). As described previously (Thomsen et al. 2012), while there was a positive correlation between YKL-40 and TG, HDL-C and TC levels, it did not correlate with LDL-C levels. It was considered that YKL-40 could have a direct or indirect lipolytic effect on adipocytes, thereby increasing plasma atherogenic lipid levels. Body mass index (BMI) was found to correlate positively with YKL-40 when adjusting for age and gender. There wasn't a significant correlation between WHR and YKL-40. YKL-40

is thought to contribute to the activation of monocytes and the formation of macrophages and their transformation into lipid-laden foam cells leading to plaque formation. YKL-40 elevated matrix metalloproteinase (MMP)-9 levels in peripheral monocytes from healthy donors (Thomsen et al. 2012). The authors showed that elevated YKL-40 was related to a 34% increase in TG levels and with a twofold increased risk of ischemic stroke. Statin treatment reduced both plasma YKL-40 and TG levels. The increased TG levels might be a cause of both high plasma YKL-40 levels and ischemic stroke (Kjaergaard et al. 2015).

It has been observed in various studies that YKL-40 is associated with IR and microvascular complications in diabetes. It is now known that patients with T2DM had increased levels of serum YKL-40 and high-sensitivity C-reactive protein (hsCRP). YK-40 was correlated with HOMA-IR and TG (Rathcke et al. 2006). In another study, the patients with T2DM had elevated plasma YKL-40, but not higher expression in adipose tissue YKL-40 mRNA. There was a significant correlation between plasma YKL-40 and fasting plasma glucose, IR, and IL-6. YKL-40 might be involved in metabolism (Nielsen et al. 2008). In a study, YKL-40 levels were elevated in patients with T1DM and independently associated with increasing levels of albuminuria. These results suggest that YKL-40 could be related to progressing vascular complications in patients with diabetes and could be a possible early marker of microvascular complications (Rathcke et al. 2009). As described previously (Catalán et al. 2011), circulating YKL-40 concentration and its VAT expression increased in obese patients with T2DM and were associated with IR and inflammation. In obese patients, high levels of YKL-40 declined after weight loss following a conventional hypocaloric diet. The study demonstrated that YKL-40 secretion via macrophages was elevated by pro-inflammatory cytokines like TNF-α and IL-1β in relation to the pathogenesis of NAFLD in vitro. In NAFLD patients, serum YKL-40 levels were elevated in line with the progression of liver fibrosis (Kumagai et al. 2016).

YKL-40's Relationship with Diet and Vitamins

Serum YKL-40 levels in food allergy (FA) patients increased and were positively correlated with blood eosinophil counts and airway allergic responses (Kim et al. 2011). YKL-40 stimulates the antigen-induced T helper 2 (Th2) response and seems to induce tissue inflammation and fibrosis mediated by IL-13. YKL-40 has an essential role in antigen sensitization and IgE induction as well as activation of innate immune cells (Rathcke and Vestergaard 2009). As described previously (Liu et al. 2019), serum YKL-40 levels were higher in non-eosinophilic asthma compared with eosinophilic asthma. YKL-40 was negatively correlated with blood eosinophils and IgE – potential biomarkers of Th2-type inflammation. Also, serum YKL-40 was positively correlated with non-T2 inflammatory markers such as IL-1ß and IL-6 (Liu et al. 2019). Serum levels of CHI3L1 were significantly high in children with FA and in an IgE-mediated FA mouse model. CHI3L1 expression was elevated in ovalbumin-treated wild-type intestinal macrophages, and it caused M2 macrophage

polarization. In this study, they showed that CHI3L1 supported FA via Th2 immune responses and M2 macrophage polarization in combination with MAPK/ERK and PI3K/AKT signaling pathways in FA. CHI3L1 also participates in tissue remodeling and apoptosis. CHI3L1 binds to the IL-13 receptor α2 and joins in a multi- meric complex with IL-13 (Th2 cytokine). IL-13 receptor α2, and transmembrane protein 219 that intercedes various CH13L1 signaling responses leading to regulate cellular and tissue responses. Their findings demonstrated that the immune response and lipid metabolism were concerned with CHI3L1-induced FA (Kim et al. 2020).

Plant-derived a-linolenic acid (ALA) may lessen the risk of CVD, likely by alleviating systemic inflammation and ameliorating endothelial function. The serum concentration of YKL-40 reduced after ALA-rich diet. The lessened serum-soluble intercellular adhesion molecule-1, sE-selectin, CRP, and YKL-40 levels were significantly correlated with reduced body fat mass. The high ALA intake led to a more reduction in the serum concentration of YKL-40 and diastolic BP compared with the intake of the low-ALA control diet. Diet-promoted body-weight loss meaningfully decreased the circulating YKL-40 concentrations in overweight-to-obese patients with MS traits (Egert et al. 2014).

The current dietary habits and lifestyles of modern societies can cause clinical disorders and excessive weight gain due to excessive energy overload and decreased physical activity. Additionally, a genetic background was thought to lead to weight gain and obesity-related symptoms. A pathway analysis was performed to identify genes differentially expressed in the subcutaneous abdominal adipose tissue (SCAAT) from the obese. Inflammation-related genes (ALCAM, CTSB, C1S, YKL-40, MIF, SAA2) increased and were associated with some MS features. YKL-40 gene expression was overexpressed in the SCAAT of the obese (González-Muniesa et al. 2013). Quercetin, a natural flavonoid, is found in many vegetables and fruits and plays an immensely important role in anti-inflammatory, antioxidant, free radical scavenging, cytoprotective, and antiallergic activities. In the study, it was now known that the levels of YKL-40 significantly diminished after taking single-dose quercetin before cecal ligation and puncture procedure in sepsis model. Quercetin also resulted in diminishing serum levels of YKL-40 and periostin in oxidative lung injury mediated by the experimental sepsis model. In this study, serum YKL-40 levels were significantly higher in the sepsis group, and quercetin treatment markedly lessened YKL-40 levels (Gerin et al. 2016). IL-6 and hypoxia trigger YKL-40 synthesis. YKL-40 is a growth factor for fibroblasts and stimulates vascular endothelial growth factor (Nøjgaard et al. 2015). The plasma concentrations of YKL-40 were significantly lower in Inuit living in Greenland compared with Inuit living in Denmark and Danes. A number of factors, including different alcohol intake patterns, nutrition, and genes, may play a role in these findings. In Danes, factors like a high alcohol intake elevated liver enzymes, and benign liver diseases are associated with high YKL-40 values. The high alcohol intake of Inuit living in Denmark and Nuuk was related to high plasma YKL-40 in a dose-dependent manner, while there was no relation between alcohol intake and YKL-40 in Inuit living in small towns and settlements in Greenland. High plasma YKL-40 was correlated with high ALP, bilirubin, AST, and low albumin (Nøjgaard et al. 2015).

As described previously (Schæbel et al. 2015), vitamin D and the inflammatory markers YKL-40 and hsCRP were significantly higher in the group fed with the traditional Greenlandic diet. YKL-40 was correlated with age, BMI, smoking, diet, and ethnicity but not by vitamin D. The traditional Greenlandic diet consists mainly of fish and marine mammals and high levels of persistent organic pollutants that limit the anti-inflammatory effect of vitamin D. This diet includes some nutrients such as vitamins A and D, Fe, I, P, Se, and n-3 fatty acids (Schæbel et al. 2015).

In a study, the patients with deficient vitamin D levels had significantly higher levels of serum IL-6, YKL-40 and CRP levels are higher in patients with vitamin D deficiency than in patients with sufficient vitamin D levels. Advanced tumor stage was related to high levels of serum biomarkers IL-6, YKL-40, and CRP, and these markers were associated with tumor inflammation (Rasmussen et al. 2021). Vitamin D, a steroid hormone which regulates calcium and phosphorus homeostasis, is very important for bone health through the intestine, bone, kidney, and parathyroid gland (Can et al. 2019). Vitamin D and its metabolites play important roles in some processes such as cellular growth and adhesion, apoptosis, inflammation, immunity, and stress responses. Therefore, vitamin D deficiency has been shown to be implicated in the pathogenesis of various chronic low-grade inflammatory conditions and diseases such as diabetes and CVD. Mostly, vitamin D deficiency is considered as plasma/serum 25 OH D level $<$ 20 ng/mL (Can et al. 2019). As described previously (Baser et al. 2015), fibrinogen levels were elevated in patients with vitamin D deficiency and decreased significantly after vitamin D treatment. Apart from that, hs-CRP levels were found to be similar in healthy controls and vitamin D-deficient patients. In patients with vitamin D deficiency, the total antioxidant status levels decreased, and the total oxidant status and ischemia-modified albumin levels increased (Baser et al. 2015). Surprisingly, YKL-40 appears to share similar roles with vitamin D in cell growth, apoptosis, angiogenesis, extracellular tissue remodeling, inflammation, and immune responses (Can et al. 2019). Vitamin D can initiate the inflammatory response by binding to VDR expressed by inflammatory cells such as neutrophils and macrophages and regulate gene transcription affecting inflammatory signaling cascades. Vitamin D suppresses pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α and increases an anti-inflammatory cytokine like IL-10. YKL-40 has been shown to be involved in the inflammatory response, and its level rises in inflammatory diseases. It has been described that YKL-40 is stimulated by pro-inflammatory cytokines such as IL-13, IL-6, IFN-γ, TNF-α, and IL-1β. As described previously (Can et al. 2019), there was a significant increase in YKL-40 levels in the group with vitamin D deficiency (Fig. 1). They suggested that there might be a relationship between vitamin D level and YKL-40. The significant increase in YKL-40 levels in the group with vitamin D deficiency may play a role in various chronic inflammatory diseases together (Can et al. 2019). Vitamin D deficiency has recently been reported to be involved in the pathophysiology of CVD development which is closely related to a chronic inflammatory condition leading to endothelial dysfunction and atherosclerosis (Mozos et al. 2015). Vitamin D deficiency was associated with IR and the development of diabetes (Clemente-Postigo et al. 2015). Also, YKL-40 levels were significantly higher in relation to inflammation in diabetes (Żurawska-Płaksej et al. 2015). Interestingly,

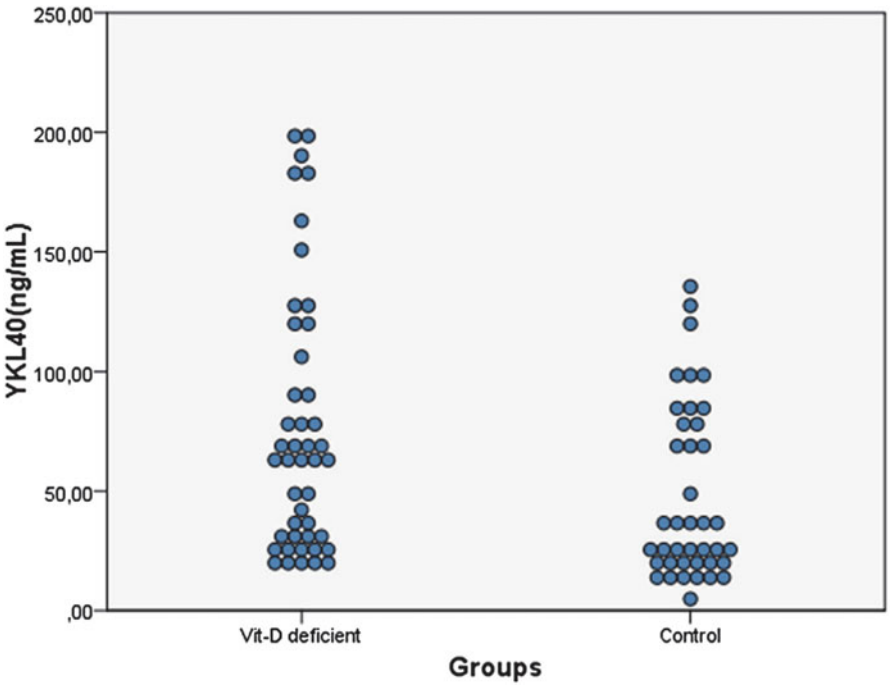

Fig. 1 Dot plot of YKL-40, representing median values of YKL-40 levels in vitamin D-deficient and control groups

very low vitamin D levels were found to be associated with the pathogenesis of acute lung injury (ALI). YKL-40 inhibited hyperoxic ALI. This suggests a cross-link between vitamin D and YKL-40 (Parekh et al. 2013; Sohn et al. 2010). As described previously (Omidian et al. 2019), vitamin D supplementation also significantly reduced serum YKL-40 and MCP-1 levels in T2DM and vitamin D deficiency. Furthermore, there was a significant decrease in IL-6, fasting insulin, and HOMA-IR after 3 months of supplementation. It was suggested that vitamin D might contribute in reducing diabetic complications via modulating YKL-40 and MCP-1 signaling pathway. All this information suggests that there may be a link between vitamin D deficiency and increased YKL-40 levels in the pathogenesis of many chronic inflammatory diseases (Omidian et al. 2019).

More studies are needed to reveal the relationship between vitamin D, nutrition, and YKL-40 and how they interact and affect each other in inflammatory processes.

It has been determined that the fruit bodies of *Morchella esculenta* (ME), a fungus, contain 21 fatty acids, 17 amino acids, and 13 minerals (Meng et al. 2019). In a mouse model of acute alcohol-induced liver injury, ME has been shown to reduce serum ALT and AST levels and serum and liver tissue acetaldehyde dehydrogenase activity. It is suggested that ME controls lipid metabolism by suppressing TG, TC, and HDL. ME suppresses the production of inflammatory

factors such as YKL-40, IL-7, plasminogen activator inhibitor type 1, and retinol-binding protein 4 in serum and/or liver tissue. ME ameliorated alcohol-induced imbalance in prooxidative and antioxidative signaling via nuclearfactor-erythroid 2-associated factor 2 (Nrf-2) by increasing superoxide dismutases1 and 2, catalase, and heme oxygenase-1 and heme oxygenase-2 in the liver. Also, ME suppressed the levels of nuclear factor-kappa B p65 (NF-kB p65) in the liver. Its antioxidative and anti-inflammatory effect may be related to the regulation of Nrf-2 and NF-kB signaling (Meng et al. 2019).

Alternative and complementary therapeutic approaches, such as the use of various herbal, nutritional, and physical manipulations, have been shown to affect YKL-40 level and alleviate OA symptoms (Arjmandi et al. 2004). In a study, patients received 40 g of supplemental soy protein (SP) or milk-based protein (MP) daily for 3 months. Especially in men, a significant increase in serum IGF-I and a significant decrease in YKL-40 serum level were detected in patients given SP compared to MP (Arjmandi et al. 2004). Serum YKL-40 and hsCRP levels of the patients with knee OA were higher than the healthy control group. After 3 months of treatment, serum YKL-40 level increased significantly in the treated hot pack group. Decreased YKL-40 level in mud pack treatment seems to slow the progression of knee OA (Güngen et al. 2012). In apolipoprotein (E)-deficient (ApoE-/-) mice fed with a high-fat diet, serum YKL-40, IL-6, TNF-α, MMP-9, plaque size, and macrophage levels in plaques were found to have increased significantly. YKL-40 can accelerate the progression of atherosclerosis through its pro-inflammation role but does not affect lipid metabolism (Chen et al. 2019).

YKL-40 in Cancers

The serum level of YKL-40 was positively correlated with the prevalence of cancers like lung, kidney, and germ cell tumors (Deng et al. 2020).

A meta-analysis of eight studies indicated that a high YKL-40 expression was independently related to worse overall survival in glioblastoma patients (Qin et al. 2017). The serum concentrations of VEGF, MMP-2 and MMP-9, and YKL-40 were significantly higher in melanoma patients (Lugowska et al. 2015). Treatment of mammary tumor-bearing mice with chitin microparticles, natural ligand for YKL-40, suppressed angiogenesis, production of pro-inflammatory mediators, YKL-40 expression, tumor growth, and metastasis (Libreros et al. 2013).

YKL-40 is a key pro-inflammatory cytokine related to the pathology of nutrition and obesity-linked cancers. In a study, increases in certain lipid profiles, YKL-40, nitric oxide, Cu, Zn, and Fe and decreases in antioxidant status and Mg were detected in women with benign tumors and breast cancer. There is also a significant positive correlation between serum YKL-40 level and TC, LDL-C, VLDL-C, and TG in the breast cancer group, although only YKL-40 and VLDL-C showed a significant positive correlation in benign tumor patients (Shahy et al. 2020). Interestingly, as described previously (Bielawski et al. 2020), there were significantly higher concentrations of YKL-40 and leptin and a lower concentration of

adiponectin in the group of patients with invasive ductal carcinoma in contrast to their invasive lobular carcinoma counterparts in non-metastatic breast cancer. Circulating YKL-40, leptin, and adiponectin levels and tissue factor activity were not related to other prognostic indicators, such as tumor grade and TNM stage or tumor size and nodal status. Also, a higher concentration of YKL-40 and adiponectin as an anti-cancer protein was detected in breast cancer patients who gave birth to one child rather than those with two or three children. Therefore, the reduction of adiponectin (an anti-inflammatory adipokine) and increment of leptin and YKL-40 in normal-weight breast cancer patients may be related to a more aggressive tumor phenotype. As regards their results, overweight breast cancer patients had a better prognosis of YKL-40, leptin, and adiponectin levels. There was an approximately threefold increased risk of disease recurrence or death for normal-weight versus obese women (Bielawski et al. 2020).

Relationship Between YKL-40 and Cardiovascular Diseases

Some authors have introduced YKL-40 as a new biomarker for CVD due to its elevated levels (Rathcke and Vestergaard 2009; Rathcke et al. 2006). As described previously (Can et al. 2015), serum YKL-40 levels were significantly higher in the first and second day of acute myocardial infarction (AMI) patients than those of the control subjects (Table 1). Serum YKL-40 levels in the first day of AMI patients also were significantly higher than those of the second day of AMI patients (Table 2). Serum YKL-40 levels at the first day and second day of AMI could be used as a clinically useful marker for diagnosis of AMI. The acute-phase protein YKL-40 is an inflammatory biomarker in both the early and late phases of the atherosclerotic process and coronary artery disease (CAD) patients and can be used for monitoring the efficiency of medical treatment of patients with CAD (Can et al. 2015). In the prognostic study involving a total of 4298 patients with stable ischemic heart disease who were followed for approximately 31 months, serum YKL-40 levels were related to increasing age, blood pressure, myocardial infarction, and cardiovascular death. These results indicated that a high level of YKL-40 could be an important predictor of adverse cardiovascular events (Kastrup et al. 2009). In patients with acute myocardial infarction, treated with primary percutaneous coronary intervention (PCI), the raised serum YKL-40 levels were diminished in the course of one month after PCI therapy (Hedegaard et al. 2010). Serum YKL-40 levels raised in Japanese patients with chronic heart failure and in patients with adverse

Table 1 Serum biomarkers of the patients and controls

	Control subjects	AMI patients – first day	AMI patients – second day	p-Value
YKL-40 (ng/mL)	37.11 ± 4.30	$69.10 \pm 16.58^{***}$	$60.64 \pm 16.01^{***}$	< 0.001

$^{***}p < 0.001$ compared with control group

Table 2 Serum biomarkers in the first and second day of AMI patients

	AMI patients – first day	AMI patients – second day	p-Value
YKL-40 (ng/mL)	69.10 ± 16.58	60.64 ± 16.01	< 0.01

cardiovascular events. It was thought that YKL-40 can be a potential predictor of adverse clinical outcomes in patients with chronic heart failure (Bilim et al. 2010). Higher serum levels of YKL-40, independent of serum CRP and fibrinogen levels, were strongly associated with an approximately twofold increased risk of atrial fibrillation on hospitalizations (Marott et al. 2013).

Applications to Prognosis and Other Diseases or Conditions

Cerebrospinal fluid (CSF) YKL-40 levels were significantly increased in Alzheimer's disease (AD) patients and were correlated with dementia biomarkers like tau proteins and amyloid beta. It was thought that CSF YKL-40 level could help in differentiating dementia types and in separating patients with mild cognitive impairment in stable phase from patients who progressed to dementia (Muszyński et al. 2017). Baran et al. showed that the plasma levels of YKL-40 were elevated in psoriatic patients, also positively associated with psoriasis area and severity index (Baran et al. 2018). Serum YKL-40 levels were raised in obstructive sleep apnea syndrome patients with coronary artery disease and significantly diminished by intensive statin treatment (Sui et al. 2013). YKL-40 increased in macrophages at the sites of allergic pulmonary inflammation. YKL-40 knock out (KO) mice, allergic pulmonary inflammation decreased due to the exaggerated expression of Fas (Lee et al. 2012).

It was shown that both serum and sputum YKL-40 levels were elevated in patients with COPD and that these high levels were significantly associated with disease status and lung function (Tong et al. 2018). Increased YKL-40 expression was detected in spleen tissues in patients with splenomegaly and was significantly related to Child-Pugh classification, free portal pressure, and splenic fibrosis. It was suggested that YKL-40 induced splenic tissue remodeling of patients with portal hypertension and might be considered a therapeutic target for splenomegaly (Wang et al. 2012). In a study, the highest levels, up to three- to fivefold increase, were found in patients with alcoholic cirrhosis. It was shown that the plasma YKL-40 levels were related to the degree of fibrosis, and these changes were independent of the etiology of the liver disease (Johansen et al. 1997). Serum YKL-40 may be used to predict the degree of liver fibrosis and to assess the efficacy of interferon (IFN) therapies in patients with HCV-linked liver disease (Saitou et al. 2005).

The patients with polymyositis/dermatomyositis had a significantly higher serum YKL-40 concentration. Myositis disease activity assessment showed a positive correlation between serum YKL-40, C-reactive protein, erythrocyte sedimentation rate (ESR), and ferritin (Gao et al. 2019). YKL-40 production greatly increased in

response to tissue damage in the early stage of chondrocyte monolayer culture and in normal cartilage explant cultures. Spontaneous release of YKL-40 was higher in OA compared to normal cartilage explant cultures. In chondrocyte monolayer cultures, levels of secreted YKL-40 were decreased by interleukin-1beta (IL-1beta) and transforming growth factor beta (TGF beta) (Johansen et al. 2001).

A significant decrease of cartilage oligomeric matrix protein (COMP) and leptin and an increase of YKL-40, adiponectin, and resistin were found in the blood of untreated juvenile idiopathic arthritis patients. Also, there was a correlation between COMP and leptin, adiponectin, and BMI and between YKL-40 and leptin, adiponectin, BMI, CRP, and ESR. Furthermore, treated patients with an active JIA showed a correlation between COMP and adiponectin and between YKL-40 and leptin, adiponectin, BMI, CRP, and ESR (Winsz-Szczotka et al. 2020).

Conclusion

Studies have shown that YKL-40 is related to extracellular matrix remodeling, many inflammatory and tissue remodeling conditions, angiogenesis, development and progression of atherosclerosis, IR, and lipid abnormalities and is effective in the pathogenesis of various diseases such as obesity, diabetes, and cancer. In addition, studies have indicated that YKL-40 is associated with nutrition, and its level has diminished through diet-promoted body-weight loss. In summary, more studies are needed to understand the link between nutritional status and YKL-40 metabolism. The data that can be obtained through extensive studies in the future could lead to the development of new treatments in order to counter the harmful effects of over-nutrition on immunity and metabolism.

Mini-dictionary of Terms

Lysophosphatidic acid (LPA): A rich source of the pleiotropic lipid mediator
YKL-40: A 40-kDa heparin- and chitin-binding glycoprotein also known as human cartilage glycoprotein 39 (HCgp39) or chitinase-3-like protein 1 (CHI3L1)
Curcuma longa (CM): A major polyphenol of turmeric
Del: The purple fruit-derived anthocyanidin

Key Facts of YKL-40

YKL-40 is a 40-kDa heparin- and chitin-binding glycoprotein.
High circulating serum YKL-40 levels have been found in many inflammatory and tissue remodeling conditions such as cancer, osteoarthritis (OA), rheumatoid arthritis, liver fibrosis in nonalcoholic fatty liver disease (NAFLD), IR, obesity, endothelial dysfunction, atherosclerosis, and CVD.

YKL-40 is related to migration, reorganization, and adhesion of endothelial cells and smooth muscle cells in angiogenesis.

YKL-40 may be a useful initial screening tool or follow-up risk indicator for lipid abnormalities, atherosclerosis, and CVD.

YKL-40 is associated with IR and microvascular complications in diabetes.

In obese patients, high levels of YKL-40 declined after weight loss following a conventional hypocaloric diet.

Diet-promoted body-weight loss meaningfully decreased the circulating YKL-40 concentrations.

YKL-40 appears to share similar roles with vitamin D in cell growth, apoptosis, angiogenesis, extracellular tissue remodeling, inflammation, and immune responses.

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The Dietary Inflammatory Index

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Methods, Indicators, and Applications to General Population

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Abstract

Inflammation is a highly complex reaction triggered by exogenous or endogenous noxious stimuli and conditions, such as infection and tissue injury. It is associated with diseases such as cancer, obesity, and atherosclerosis.

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Studies have shown that several risk factors can play a role in stimulating inflammation, such as overweight, physical inactivity, and diet. The dietary inflammatory index (DII) is one of the indicators used to evaluate the quality of a diet based on its inflammatory capacity. This international index was presented to assess pro- and anti-inflammatory properties of an individual's diet including macronutrients, vitamins, minerals, flavonoids, and special nutrients.

Several studies have reported a significant association between DII score and diseases such as asthma, ulcerative colitis, cardiovascular disease, and metabolic syndrome. The chapter goes on to discuss the association between the DII score and cardiovascular diseases and cancer – two major causes of death in the world.

Keywords

Diet; Inflammation · Interleukin-6 · Cardiovascular diseases · Cancer

Abbreviations

CVDs	Cardiovascular diseases
DII	Dietary inflammatory index
Hs-CRP	High-sensitivity C-reactive protein
IHD	Ischemic heart disease
IL	Interleukin
MI	Myocardial infarction
TNF-α	Tumor necrosis factor-α
VCAM	Vascular cell adhesion molecule

Introduction

Inflammation is a highly complex reaction triggered by exogenous or endogenous noxious stimuli and conditions, such as infection and tissue injury in the vascularized connective tissue. It ultimately leads to increased blood flow and vascular permeability that caused the accumulation of fluid and leukocytes in the extravascular tissue, neutralization of the injurious agent, and repair of the injured tissue in the acute phase (Gallin et al. 1992; Marshall and Haskard 2002; Medzhitov 2008; Springer 1995).

Significant progress has been made in understanding the cellular and molecular events involved in the acute inflammatory response to infection and, to some extent, tissue damage. In addition, the events leading up to chronic local inflammation, especially in chronic infections and autoimmune diseases, are somewhat understandable. However, very little is known about the causes and mechanisms of chronic systemic inflammation occurring in a wide range of diseases including type 2 diabetes and cardiovascular disease. These chronic inflammatory states do not appear to be caused by the classic inflammatory stimuli: infection and injury. Instead, they appear to be associated with tissue dysfunction: that is, with a homeostatic imbalance of one of several physiological systems that are not functionally directly related to host defense or tissue repair (Medzhitov 2008). Low-grade chronic inflammation

that occurs in the absence of normal negative feedback on acute inflammation is characterized by a slight increase in the concentration of circulating inflammatory markers, including high-sensitivity C-reactive protein (hs-CRP) (Danesh et al. 1998), interleukin (IL)-6 (Ridker et al. 2000), tumor necrosis factor-α (TNF-α) (Blaser et al. 2016), and vascular cell adhesion molecule (VCAM)-1 (Kolb and Mandrup-Poulsen 2005; Lee et al. 2009). This condition has been shown to be associated with diseases such as depression, some cancers such as colon and rectum cancer, higher waist circumference and body mass index, and a key component in the process of atherosclerosis, which is involved in cardiovascular diseases such as coronary heart disease, myocardial infarction, and ischemic stroke (Arbel et al. 2012; Blake and Ridker 2002; Howren et al. 2009; Laukoetter et al. 2011; Pearson et al. 2003; Ridker et al. 2002; Wood et al. 2012).

Studies have shown that several risk factors can play a role in stimulating inflammation, such as overweight, physical inactivity, and diet (Calder et al. 2011; Petersen and Pedersen 2005). Numerous studies on diet, as a modifiable risk factor in the development of inflammation, have shown that consumption of diets such as the Mediterranean diet, which is rich in whole grains, olive oil, fruits, vegetables, and fish, can reduce the level of inflammatory factors. In particular, it seems that the intake of fruits and vegetables can reduce inflammatory factors such as CRP, IL-6, and TNF-a (Estruch et al. 2006). On the other hand, Western diets which are high in pro-inflammatory foods, including high-sugar foods (especially desserts and soft drinks), refined grains, red and processed meats, and foods high in saturated and trans fatty acids and sodium, increase the levels of inflammatory biomarkers in the blood such as CRP and IL-6 (Johansson-Persson et al. 2014; King et al. 2003). According to studies, certain nutrients can also reduce the level of inflammation. One of the strongest fighters in inflammation is n-3 polyunsaturated fatty acid (PUFA), which can prevent cell damage by increasing membrane fluidity (Ferrucci et al. 2006). Also, vitamins such as vitamin E (Bertran et al. 2005), vitamin C (Wannamethee et al. 2006), and β-carotene have antioxidant and anti-inflammatory properties (Erlinger et al. 2001).

Many studies have evaluated the quality of the diet based on a number of different indicators (Haines et al. 1999; Puchau et al. 2009). The dietary inflammatory index (DII) is one of the indicators created by researchers at the University of South Carolina which used to evaluate the quality of a diet based on its inflammatory capacity (Cavicchia et al. 2009; Hebert et al. 1997; Shivappa et al. 2014). This relatively new index is a literature-based scoring system, focuses on diet and inflammation, and is standardized for the average world diet, which helps reduce the deficiencies of previous dietary indices. The purpose of presenting this International Index in 2009 was to assess pro- and anti-inflammatory properties of an individual's diet including macronutrients, vitamins, minerals, flavonoids, and special nutrients. This index, which was updated in 2014, has previously been shown to be associated with inflammation, especially CRP, IL-6, and TNF-α levels in adults, and it seems to be used in treatment programs as well (Cavicchia et al. 2009; Galas et al. 2014; Ruiz-Canela et al. 2015; Shivappa et al. 2014). Thus far, several studies have reported a significant association between DII score and diseases such as asthma, ulcerative colitis, cardiovascular disease, and metabolic syndrome (Bodén et al. 2017; Kim et al. 2018; Shivappa et al. 2016c; Wood et al. 2015).

The DII Score Calculation

The DII® is a tool that quantifies the inflammatory degree of diet from maximally anti-inflammatory to maximally pro-inflammatory. The procedure used to develop this index has been described in detail elsewhere (Shivappa et al. 2014). Briefly, 1943 relevant papers which assessed the effect of 45 dietary factors on IL-1β, IL-4, IL-6, IL-10, TNF-α, and CRP were read and scored. Each dietary factor with pro-inflammatory effect is assigned a score of +1, −1 scored to anti-inflammatory dietary items, and 0 scored to dietary factors without any effect on the inflammatory biomarkers.

At first, to measure the DII score for each individual, the amount reported for dietary factors initially was linked to the regional representative global database that provided a robust estimate of a mean and standard deviation for each of them. To obtain the z-score, we subtracted the value of global mean intake of each dietary factor from the reported amount consumed (Table 1). Then, we divided this number by world standard deviation. Global daily mean intake and standard deviation for each dietary factor were obtained via data sets of 11 countries. To minimize the effect of "right skewing," the dietary factor-specific z-scores were converted to a proportion (i.e., with values from 0 to 1). Afterward, to create a symmetrical distribution with a range of −1 to +1 and centered on 0, the achieved value of each dietary factor was multiplied by 2 and then 1 was subtracted from it. The resulting value of each dietary factor was multiplied by an overall dietary factor-specific inflammatory effect score to achieve dietary factor-specific DII score. Finally, all dietary factor-specific DII scores were summed to calculate the DII score for each participant. The final range of the DII score is from −8.87 to 7.98, and the lower value of the DII score represents the lower dietary inflammation and the higher DII score showed the higher dietary inflammation. To control the effect of total energy intake, we adjusted all dietary factors for energy via the residual method (Willett et al. 1997) and then used the DII calculation.

The DII Score and Diseases

DII Score and Cardiovascular Diseases

Cardiovascular diseases (CVDs), containing cerebrovascular disease, myocardial infarction (MI), stroke, heart failure, ischemic heart disease (IHD), circulatory disorders, and other kinds of heart and vascular diseases, are the major cause of decreased quality of life and mortality worldwide (Fernandes and Collaborators 2018; Kyu et al. 2018). CVDs were responsible for 12.5% of deaths globally during the past decade (Wang et al. 2016), in which most of them occurred in low- and middle-income countries (Fuster 2014; Mensah et al. 2014; Yusuf et al. 2001). Crucial risk factors of CVDs consist of obesity, diabetes mellitus, high blood pressure, smoking, chronic inflammation, and high cholesterol levels (Joseph et al. 2017). Among these, chronic inflammation has been identified as the most important factor in the initiation and progression of CVDs such as atherosclerosis (Moore and Tabas 2011; Steinberg and Witztum 2010). Immunity cells release pro-inflammatory cytokines, including IL-1, IL-6, and hs-CRP, which

Table 1 Food parameters included in the dietary inflammatory index, inflammatory effect scores, and intake values from the global composite data set

Food parameter	Weighted number of articles	Raw inflammatory effect score[a]	Overall inflammatory effect score[b]	Global daily mean intake[c] (units/d)	SD[c]
Alcohol (g)	417	−0.278	−0.278	13.98	3.72
Vitamin B12 (μg)	122	0.205	0.106	5.15	2.70
Vitamin B6 (mg)	227	−0.379	−0.365	1.47	0.74
β-Carotene (μg)	401	−0.584	−0.584	3718	1720
Caffeine (g)	209	−0.124	−0.110	8.05	6.67
Carbohydrate (g)	211	0.109	0.097	272.2	40.0
Cholesterol (mg)	75	0.347	0.110	279.4	51.2
Energy (kcal)	245	0.180	0.180	2056	338
Eugenol (mg)	38	−0.868	−0.140	0.01	0.08
Total fat (g)	443	0.298	0.298	71.4	19.4
Fiber (g)	261	−0.663	−0.663	18.8	4.9
Folic acid (μg)	217	−0.207	−0.190	273.0	70.7
Garlic (g)	277	−0.412	−0.412	4.35	2.90
Ginger (g)	182	−0.588	−0.453	59.0	63.2
Fe (mg)	619	0.032	0.032	13.35	3.71
Mg (mg)	351	−0.484	−0.484	310.1	139.4
MUFA (g)	106	−0.019	−0.009	27.0	6.1
Niacin (mg)	58	−1.000	−0.246	25.90	11.77
n-3 Fatty acids (g)	2588	−0.436	−0.436	1.06	1.06
n-6 Fatty acids (g)	924	−0.159	−0.159	10.80	7.50
Onion (g)	145	−0.490	−0.301	35.9	18.4
Protein (g)	102	0.049	0.021	79.4	13.9
PUFA (g)	4002	−0.337	−0.337	13.88	3.76
Riboflavin (mg)	22	−0.727	−0.068	1.70	0.79
Saffron (g)	33	−1.000	−0.140	0.37	1.78
Saturated fat (g)	205	0.429	0.373	28.6	8.0
Se (μg)	372	−0.191	−0.191	67.0	25.1
Thiamin (mg)	65	−0.354	−0.098	1.70	0.66
Trans fat (g)	125	0.432	0.229	3.15	3.75
Turmeric (mg)	814	−0.785	−0.785	533.6	754.3
Vitamin A (RE)	663	−0.401	−0.401	983.9	518.6
Vitamin C (mg)	733	−0.424	−0.424	118.2	43.46
Vitamin D (μg)	996	−0.446	−0.446	6.26	2.21
Vitamin E (mg)	1495	−0.419	−0.419	8.73	1.49

(continued)

Table 1 (continued)

Food parameter	Weighted number of articles	Raw inflammatory effect score[a]	Overall inflammatory effect score[b]	Global daily mean intake[c] (units/d)	SD[c]
Zn (mg)	1036	−0.313	−0.313	9.84	2.19
Green/black tea (g)	735	−0.536	−0.536	1.69	1.53
Flavan-3-ol (mg)	521	−0.415	−0.415	95.8	85.9
Flavones (mg)	318	−0.616	−0.616	1.55	0.07
Flavonols (mg)	887	−0.467	−0.467	17.70	6.79
Flavonones (mg)	65	−0.908	−0.250	11.70	3.82
Anthocyanidins (mg)	69	−0.449	−0.131	18.05	21.14
Isoflavones (mg)	484	−0.593	−0.593	1.20	0.20
Pepper (g)	78	−0.397	−0.131	10.00	7.07
Thyme/oregano (mg)	24	−1.000	−0.102	0.33	0.99
Rosemary (mg)	9	−0.333	−0.013	1.00	15.00

RE retinol equivalents

[a]This is referred to as the "food parameter-specific raw inflammatory effect score" abbreviated here for ease of presentation. Note that the effect is per unit amount noted for each food parameter

[b]This refers to the "food parameter-specific overall inflammatory effect score" accounting for the robustness of the literature, which is considered optimal at the median of 236 articles

[c]From the world composite database

have a key role in the formation of atherosclerotic plaques. Moreover, in patients with MI and unstable angina the level of inflammatory biomarkers increased (Hansson 2005). Diet has a key role in chronic inflammation development and different studies have evaluated the effect of different food consumption in the production of pro-inflammatory biomarkers (Emerson et al. 2017; Galland 2010). Higher intake of red and processed meat, trans and saturated fatty acids, refined grains, as well as sodium is associated with increased chronic inflammation (King et al. 2003; Van Bussel et al. 2013), whereas greater consumption of olive oil, fish, vegetables and fruits, nuts, and cereals (the Mediterranean diet) decreases the concentrations of IL-6 and hs-CRP (Esposito et al. 2004; Serrano-Martinez et al. 2005). Foods with anti-inflammatory properties such as vegetables, fruits, fish, and cereals prevent from CVDs (Esposito and Giugliano 2006; Griffiths et al. 2016). In 2014, Shivappa and his colleagues designed the DII score to evaluate anti-inflammatory and pro-inflammatory properties of diet and after that found out the association between this score and different disorders such as CVDs (Shivappa et al. 2014).

The relationship between the DII score and CVDs morbidity has been assessed in different investigations. In a large study on 7743 French adults, greater adherence to pro-inflammatory diet was associated with higher risk of MI (Neufcourt et al. 2016). Additionally, the results of a cross-sectional study suggested that the higher DII

score was related to combined circulatory disorders, congestive heart failure, heart attack, and stroke (Wirth et al. 2016). Boden et al. also in a case-control study on 1389 cases of first MI and 5555 controls showed higher pro-inflammatory diet consumption was associated with increased risk of MI (Bodén et al. 2017). Besides, in a large cohort study carried out on 7216 elderly people, the findings demonstrated that after 4.8 years of follow-up, people with a higher DII score had a greater risk for CVD (Garcia-Arellano et al. 2015). Furthermore, in 2015, O'Neil et al. in an Australian investigation showed the group consuming a more pro-inflammatory diet had a higher risk for CVDs in comparison to the group consuming a more anti-inflammatory diet (O'Neil et al. 2015). Moreover, in a cohort study conducted on 18,794 Spanish adults with a mean age of 38 years, individuals with higher adherence to pro-inflammatory diet had a higher risk of CVD (Ramallal et al. 2015).

The association between the DII score and CVD mortality has been evaluated in various studies. In a cohort study conducted on 1304 women over 70 years old, participants with the most pro-inflammatory DII score had an increased risk of IHD and atherosclerotic vascular disease- related mortality (Bondonno et al. 2017). Moreover, in another investigation of American adults, individuals with the most pro-inflammatory properties had a higher risk of CVD mortality (Deng et al. 2017). Shivappa et al. in a large study on 28,677 American women showed that participants following a pro-inflammatory diet had a higher risk of CVD and coronary heart disease mortality in comparison to participants following an anti-inflammatory diet (Shivappa et al. 2016a).

The DII Score and Cancer

Cancer is one of the main reasons for global mortality and different risk factors may contribute to the development of this crisis. Chronic inflammation is one of the most leading risk factors involved in the pathogenesis of this disease (Touvier et al. 2013). Inflammatory and epithelial cells produce reactive oxygen/nitrogen species under chronic inflammation and these substances cause DNA mutations, resulting in the development of cancer (Murata et al. 2012; Pinlaor et al. 2004). Diet has a key role in chronic inflammation development and different studies have evaluated the effect of different food consumption in the production of pro-inflammatory biomarkers (Emerson et al. 2017; Galland 2010). Higher intake of red and processed meat, trans and saturated fatty acids, refined grains, as well as sodium is associated with increased chronic inflammation (King et al. 2003; Van Bussel et al. 2013), whereas greater consumption of olive oil, fish, vegetables and fruits, nuts, and cereals (the Mediterranean diet) decreases the concentrations of IL-6 and hs-CRP (Esposito et al. 2004; Serrano-Martinez et al. 2005). Reduction in the level of pro-inflammatory biomarkers due to higher adherence to the Mediterranean diet leads to the prevention of tumorigenesis and cancer (Ostan et al. 2015). To assess the total inflammatory properties of diet and evaluate the association between these properties and different disorders such as cancer, the DII score was designed by Shivappa and his colleagues (Shivappa et al. 2014).

The relationship between the DII score and risk of cancer was evaluated in different studies. In a case-control study of 230 individuals with gastric cancer and 547 controls

in Italy, the findings suggested that greater adherence to pro-inflammatory diet was associated with higher risk of gastric cancer (Shivappa et al. 2016b). Moreover, in an investigation of 923 Korean patients with colorectal cancer and 1846 controls, the higher DII score was related to higher risk of colorectal cancer (Cho et al. 2016). In addition, Shivappa et al. found out the positive association between pro-inflammatory diet consumption and risk of prostate cancer among Italian men (Shivappa et al. 2015a). Furthermore, the results of a study of pancreatic cancer patients demonstrated that a more pro-inflammatory diet was related to higher risk of pancreatic cancer compared to more anti-inflammatory diet (Shivappa et al. 2015b).

Additionally, various investigations assessed the association between the DII score and cancer mortality. In a large cohort study on 122,788 postmenopausal women, higher risk of death from breast cancer was associated with higher DII score at baseline of the study (Tabung et al. 2016). Moreover, in 2016 in a prospective investigation on 13,280 adults, individuals following a pro-inflammatory diet have higher risk of death from all cancers than the ones that consume anti-inflammatory diet (Deng et al. 2017).

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, we discussed a relatively new indicator called the dietary inflammatory index (DII) score. The DII score was the first try to quantify the overall effect of diet on inflammatory situation. The DII score classifies the degree of inflammation individuals' diet on a continuum from maximally anti-inflammatory to maximally pro-inflammatory diet. In this index, each dietary factor with pro-inflammatory effect is assigned a score of +1, -1 scored to anti-inflammatory dietary items, and 0 scored to dietary factors without any effect on the inflammatory biomarkers. Research has shown that the DII score can predict interval changes in IL-1β ، IL-4 ، IL-6 ، IL-10 ، TNF-α and CRP (Shivappa et al. 2014). It seems that chronic inflammation is an important factor in the onset of many diseases worldwide such as cancer, cardiovascular diseases, metabolic syndrome, type 2 diabetes mellitus, psychological disorders, osteoporosis and abdominal obesity in children and adolescents that the DII score can predict this status (Aslani et al. 2019; Aslani et al. 2020; Zahedi et al. 2018; Abdurahman et al. 2018; Zahedi et al. 2020; Aghababayan et al. 2020; Fu et al. 2021; Fang et al. 2020; Salari-Moghaddam et al. 2021).

Conclusion

Diet is one of the modifiable risk factors for many diseases that can play an important role in the prevention of chronic diseases. Previous studies have shown that the DII as a useful tool can show the effect of food on the inflammation and predict many inflammatory diseases such as cancer, cardiovascular disease, and psychological disorders. By making this issue a priority for public health and nutrition education, we can try to prevent diseases in healthy people and to improve the health and quality of life of people in the community (Fig. 1).

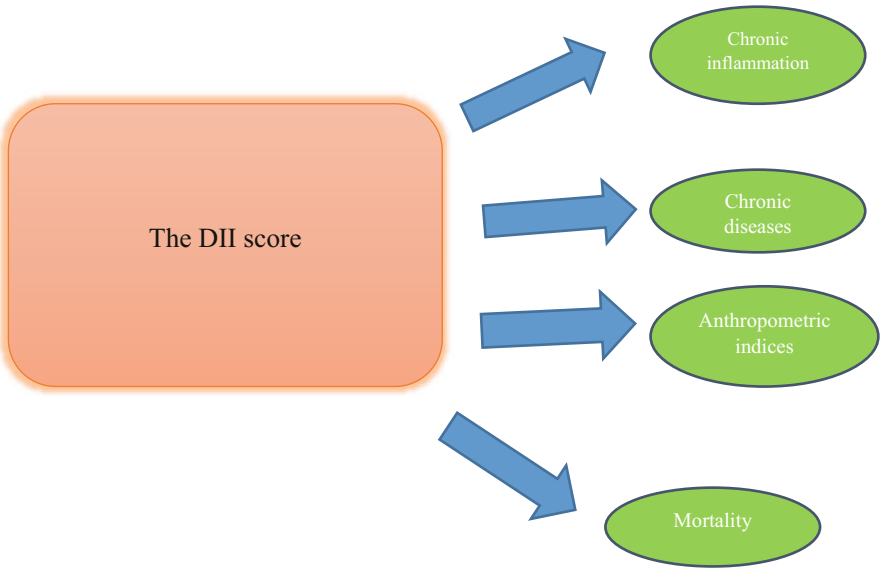

Fig. 1 Association between the DII score and some parameters related to quality of life

Summary Point

- Diet can play an important role in many diseases associated with the inflammatory system by affecting inflammation.
- The DII score is a tool to assess the degree of inflammation in the diet, which takes into account the whole diet.
- The DII score classifies food components from pre-inflammatory to anti-inflammatory due to their effect on inflammatory biomarkers, includig interleukins.
- The results of various studies have shown that higher DII score could be associated with an increased risk of cardiovascular disease such as congestive heart failure, heart attack, and stroke.
- The relationship between the DII score and risk of cancer has been studied and the results have shown that pro-inflammatory diets can increase the risk of developing cancers including colorectal cancer and pancreatic cancer and the mortality of cancers.

Mini-dictionary of Terms

- **Cancer:** Cancer is one of the causes of illness and death in the world due to mutations in DNA caused by a variety of factors, including inflammation.
- **Cardiovascular disease:** CVD consists of a wide range of disorders, including diseases of the cardiac muscle, vascular system, and stroke, which is one of the leading causes of death.

- **Chronic inflammation:** As the duration of inflammation in the body increases, humoral and cellular immune responses are produced at the site of injury, which is called chronic inflammation.
- **Dietary inflammatory index:** An indicator to measure the effect that food has on inflammation. It can be a predictor of inflammatory diseases.
- **Inflammation:** A reaction that results in tissue damage, part of which is the production of inflammatory mediators.

Key Facts of DII

- The inflammatory capacity of food components during specific period can be measured by dietary inflammatory.
- DII is a practical index for evaluating the dietary inflammatory index of foods in various age groups and countries.
- DII scored foods as anti-inflammatory with a score of −1 and pro-inflammatory with a score of +1.
- Diets with a higher inflammatory index are associated with a higher risk of inflammatory diseases.

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Thiol-Disulfide Homeostasis as an Oxidative Stress Indicator

38

Applications to Nutrition

Hayrullah Yazar, Yıldırım Kayacan, and Özcan Erel

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Abstract

Undoubtedly, the increase in the incidence of many chronic diseases, especially metabolic syndrome, is related to an unhealthy diet. "Natural-organic nutrition," which is one of the important elements of a healthy life, will also minimize the entry of "exogenous free radicals" into the body, which are accepted as oxidative stress factors in human metabolism. Recently, the thiol-disulfide balance has been accepted as an indicator for oxidative stress, which is the root cause of most diseases. Among these systemic diseases, metabolic syndrome, cancer, rheumatoid arthritis, type II diabetes, Parkinson's, Alzheimer's, cardiovascular diseases, migraine, liver, and kidney failure are the first ones that come to mind. Many factors, including obesity, oxidative stress and unhealthy diet are involved in the etiopathogenesis of many diseases. In this section, the relationship between nutrition and thiol-disulfide homeostasis, which is one of the oxidative stress markers, is presented.

Keywords

Oxidative stress · Free radicals · Genetically modified organisms · Healthy life · Organic nutrition · Obesity · Thiol-disulfide homeostasis

Abbreviations

AA	Acute appendicitis
Cd	Cardiovascular diseases
Cr	Circadian rhythm
Crf	Chronic renal failure
Dm	Diabetes mellitus
Dn	Diabetic nephropathy
Fr	Free radicals
GM	Gut microbiome
Gmo	Genetically modified organisms
HL	Healthy life
IF	Intermittent fasting
L	Lifestyle
Lbp	Lipopolysaccharide-binding protein
Nos	Nitric oxide synthase
Ntl	Native thiol
O	Obesity
On	Organic nutrition
Os	Oxidative stress
Ra	Rheumatoid arthritis
S	Sleep

Tdh	Thiol-disulfide homeostasis
Ttl	Total thiol
WHO	World Health Organization

Introduction

Thiols belong to the group of organic compounds containing a sulfhydryl group (–SH). Thiols (RSH) form disulfide (RSSR) bonds by entering the oxidation reaction. The disulfide bond is also called the disulfide bridge. Oxidative stress conditions can lead to reversible mixed disulfide formation. The disulfide bonds formed can be reduced back to thiol groups. As a result, the dynamic thiol-disulfide balance is preserved. This equilibrium state has many critical importance in cellular mechanisms, including antioxidant preserving. In addition, we should point out that the thiol-disulfide balance is associated with many diseases with increasing frequency. Among these, the first ones that come to mind are diabetes (Ateş et al. 2016a; Ozler et al. 2016), hypoparathyroidism (Or et al. 2020), cardiovascular diseases (Acar Atılgan et al. 2021; Altiparmak et al. 2016; Ateş et al. 2016b; Kundi et al. 2015; Yazar et al. 2020), malignancies (Guney et al. 2016), pneumonia (Temel et al. 2019), and urolithiasis (Sonmez et al. 2019). Also, from migraine (Alagoz et al. 2020; Gumusyayla et al. 2016a) to Alzheimer's disease (Gündüztepe et al. 2020), from eye diseases (Gulpamuk et al. 2020) to viral infections (Dertli et al. 2018), including the COVID-19 pandemic (Mete et al. 2021), there is significant clinical and experimental research (Table 1). Therefore, the determination of dynamic thiol-disulfide homeostasis can provide valuable information about a variety of normal or abnormal biochemical processes (Fig. 1). The newly developed method primarily measures native thiol (NTL, μmol/L) and total thiol (TTL, μmol/L) test kits directly from blood serum or plasma with automatic analyzers. Other test parameters from the NTL and TTL values were obtained. By calculating reduced thiol status, reduced thiol ratio, oxidized thiol (disulfide) ratio, and thiol oxidation-reduction ratio, a total of six parameters are revealed. With these data, dynamic thiol-disulfide homeostasis is assessed, and oxidative stress status is determined (Erel and Neselioglu 2014).

The dynamic thiol-disulfide homeostasis between thiol(–SH) and disulfide(–S–S–) in the human body is accepted as an indicator for oxidative stress, which is implicated in the etiopathogenesis of many diseases.

Features of Kits Used in Thiol-Disulfide Homeostasis Measurement

Analytical Recovery

The percent recovery of the novel method was determined via the addition of 200 μM oxidized glutathione to plasma samples. The mean percent recovery was 98–100%.

Table 1 Some examples of our studies on the relationship between oxidative stress and thiol-disulfide homeostasis (WOS indexing)

Title of the research	FTR
Dynamic thiol/disulphide homeostasis in patients with hypertrophic cardiomyopathy (Sari et al. 2021), https://pubmed.ncbi.nlm.nih.gov/31820030/ The effects of amantadine on lung tissue in lower limb ischemia/reperfusion injury model in rats (Orhan et al. 2021), https://pubmed.ncbi.nlm.nih.gov/33768984/ Evaluation of dynamic thiol/disulphide homoeostasis in acute ischemic stroke (Acar Atılgan et al. 2021), https://www.actamedicamediterranea.com/archive/2021/medica-1 New oxidative stress markers useful in the diagnosis of acute appendicitis in children: thiol/disulfide homeostasis and the asymmetric dimethylarginine level (Elmas et al. 2020), https://pubmed.ncbi.nlm.nih.gov/29135899/	CR. P: 119, C: 52 ER CR, P:30, C:30 CR, P:45, C:35
Oxidative stress response to different exercise intensity with an automated assay: thiol/disulphide homeostasis (Kayacan et al. 2019a), https://pubmed.ncbi.nlm.nih.gov/31409146/	ER
A new oxidative stress indicator: effect of 5-hydroxytryptophan on thiol-disulfide homeostasis in exercise (Kayacan et al. 2019b), https://pubmed.ncbi.nlm.nih.gov/30939385/	ER
A novel biomarker explaining the role of oxidative stress in exercise and l-tyrosine supplementation: thiol/disulphide homeostasis (Kayacan et al. 2018), https://pubmed.ncbi.nlm.nih.gov/29020830/	ER
Evaluation of the level of thiol-disulphide homeostasis in patients with mild and severe preeclampsia (Yuvaci et al. 2016), https://pubmed.ncbi.nlm.nih.gov/27939489/	CR, P:62, C:37

WOS Web of Science, *FTR* feature of the research, *ER* experiment on rats, *CR* clinical research, *P* patient, *C* control

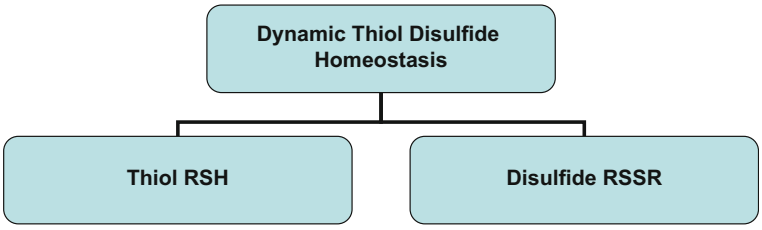

Fig. 1 Dynamic thiol-disulfide homeostasis

Linearity

The linearity of the native thiol measurement was the same with that of Ellman's reagent assay. Serial dilutions of the glutathione solution were generated. The upper limit of the linearity for the native thiol measurement was 4000 μM. Linearity of the total thiol measurement was also dependent on the amounts of NaBH4 and formaldehyde concentrations. Serial dilutions of the oxidized glutathione solution were

also generated. The upper limit of the linearity for the disulfide measurement was 2000 μM. Dilution of plasma samples did not affect the novel assay.

Lower Detection Limit

The detection limit of the assay was determined by evaluating the zero calibrator ten times. The detection limit, defined as the mean value of zero calibrator +3 standard deviations (SDs), was 2.8 μM.

Analytical Sensitivity

As the slope of the calibration line, analytical sensitivity was found to be 7.9×10^{-4} Absorbance/Amount, [A × (μM) − 1].

Interference

It was found that hemoglobin, EDTA, citrate, and oxalate did not interfere with the assay developed, but bilirubin did negatively interfere with the assay. Lipemic and uremic plasma samples did not interfere with the assay. Plasma and serum samples can be used as samples.

Precision

To determine the precision of the novel assay, we assayed three levels of a plasma pool. A plasma pool that had high disulfide levels was obtained from the samples of patients with diabetes mellitus. The plasma pool with medium disulfide levels was obtained from the samples of healthy persons. The plasma pool with low disulfide levels was obtained from the samples of patients with urinary bladder cancer. Percent coefficient variation (%CV) was 4 ($\overline{X} = 29.12$ and $\sigma X = 1.2$) for high levels, 5 ($\overline{X} = 16.03$ and $\sigma X = 0.79$) for medium levels, and 13 ($\overline{X} = 7.15$ and $\sigma X = 0.98$) for low levels.

Storage

Storage at 4 °C for 1 day led to a 7% decrease in the native thiol amount and 170% increase in the disulfide amount (total thiol, native thiol, and disulfide levels of fresh and stored plasma samples were 391, 357, and 17 μmol/L and 391, 333, and 29, respectively). Plasma native thiol, total thiol, and disulfide concentrations were not affected by storage at −80 °C for 3 months.

Thiol(–SH)-Disulfide(–S–S–) Homeostasis Test Profile

- Native thiol status(–SH)
- Dynamic disulfide status(–S–S–)
- Total (oxidized and reduced) thiol status(–SH + –S–S–)
- Reduced thiol ratio [(–SH)/(–SH + –S–S–)] × 100
- Oxidized thiol (disulfide) ratio [(–S–S–)/(–SH + –S–S–)] × 100
- Thiol oxidation reduction ratio [[(–SH)/(–S–S–)] × 100

The dynamic disulfide level was calculated as half of the difference between the total and native thiol levels. The disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol ratios were calculated after the dynamic disulfide, native thiol, and total thiol levels had been determined.

In this study, total thiol (μmol/L), native thiol (μmol/L) and disulfide levels, disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol ratios were quantified in the patient and control groups. Dynamic thiol-disulfide homeostasis in the serum samples has been identified by using an automated method newly developed by Erel et al. (Erel and Neselioglu 2014). Total thiol(–SH + –S–S–) and native thiol(–SH) concentrations in the samples were measured by using Ellman's and modified Ellman's reagent. Native thiol content was subtracted from total thiol content, and half of this difference gave the amount of dynamic disulfide bonds(–S–S–). In addition, the(–S–S–) × 100/(–SH),(–S–S–) × 100/(–SH + –S–S–), and –SH × 100/(–SH + –S–S–) ratios were calculated using these parameters.

Principle of the Assay

Reducible disulfide bonds were reduced to form free functional thiol groups. Unused reductant sodium borohydride was consumed and removed with formaldehyde, and all thiol groups including reduced and native thiol groups were determined after the reaction with 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB). Half of the difference between the total thiols and the native thiols was recorded as the dynamic disulfide amount. After the native thiols (SH) and total thiols were determined, disulfide (SS) amounts, disulfide/total thiol percent ratios (SS/SH + SS), disulfide/native thiol percent ratios (SS/SH), and native thiol/total thiol percent ratios (SH/SH + SS) were calculated (Erel and Neselioglu 2014).

Application Parameters of the Assays

Sample volume: 10 μL
R1 volume (for total –SH): 10 μL
R1′ volume (for native –SH): 10 μL
R2 (2′) volume: 110 μL
R3 (3′) volume: 10 μL

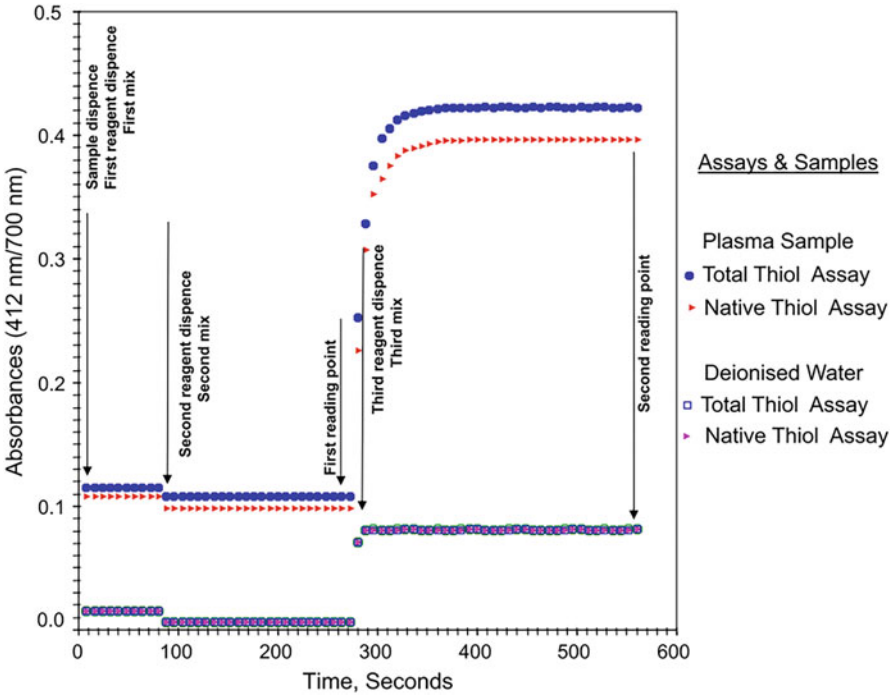

Fig. 2 Reaction kinetics of the assays

Wavelength (main wavelength): 415 nm, secondary wavelength 700 nm (optionally bichromatic) (Fig. 2)

Reading point: End-point, increasing measurement; the first absorbance is taken before the mixing of R2 and R3, and the last absorbance is taken when the reaction trace draws a plateau (assay duration is about 10 min).

Calibration type: Linear

The disulfide parameter is a value which can be calculated automatically as half of the difference of the two measured values. The assays can also be performed by manually using spectrophotometers or multiwell readers. All volumes of the samples and reagents must be increased at the same ratio. Use of a second (side) wavelength is optional.

Free Radicals and Nutrition

Free radicals (e.g., superoxide, nitric oxide, and hydroxyl radicals) and other reactive species (e.g., hydrogen peroxide, peroxynitrite, and hypochlorous acid) are produced in the body mainly as a result of aerobic metabolism. Antioxidants (e.g., glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C,

vitamin A, and tea polyphenols) and antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidases) show synergistic actions in cleaning free radicals. There has been increasing evidence over the past three decades to suggest that malnutrition (e.g., dietary deficiencies of protein, selenium, and zinc) or an excess of certain nutrients (e.g., iron and vitamin C) leads to oxidation of biomolecules and cell damage. Much of the literature supports the idea that dietary antioxidants are beneficial radioprotectants and play an important role in the prevention of many human diseases (e.g., cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration, and diabetes) (Fang et al. 2002). The imbalance between ROS production and elimination in favor of its homeostasis has been termed "oxidative stress," which has specific consequences for cell physiology. On the other hand, although almost 30 years have passed since the first definition of oxidative stress by Helmut Sies, an accepted classification of oxidative stress has not been made until today. However, it is recommended to classify oxidative stress according to its intensity to fill this gap. Therefore, oxidative stress can be classified as basal oxidative stress (BOS), low-intensity oxidative stress (LOS), intermediate-intensity oxidative stress (IOS), and high-intensity oxidative stress (HOS). Another classification of potential interest can distinguish three categories such as mild oxidative stress (MOS), temperate oxidative stress (TOS), and finally severe (strong) oxidative stress (SOS) (Lushchak 2014). In addition, the effects of free radicals, which trigger oxidative agents such as reactive oxygen species and reactive nitrogen, on oxidative stress caused by exercise should not be ignored. In this context, research on exercise physiology should also be associated with the subject (Kayacan et al. 2019a, b). Thus, thiol-disulfide homeostasis, a new marker of oxidative stress, was investigated in a study in which treadmill exercises of different intensities were examined in rats. In the study, male albino Wistar rats were randomly divided into four groups as control (CNT), low-intensity exercise (LEx), moderate-intensity exercise (MEx), and high-intensity exercise (HEx) groups and exercised for 4 weeks. After completion of the experimental protocol, serum total thiol, native thiol, and disulfide concentrations were determined using a new automated measurement method. According to the findings, it was shown that moderate-intensity exercise was more effective in reducing oxidative stress than low- and high-intensity exercise (Kayacan et al. 2019a, b). While evaluating the thiol-disulfide balance, which is a reflection of free radical effects in humans, of course, the functions of glutathione in metabolism cannot be ignored. Glutathione is a simple sulfur compound consisting of three amino acids, and it is not a protein. The functions of glutathione are very diverse but particularly include redox-homeostatic buffering. Glutathione status is modulated by dietary and other factors as well as oxidants and can affect protein structure and activity through changes in thiol-disulfide balance. For these reasons, glutathione is a converter that integrates environmental information into the cellular network. While the mechanic details of this function remain to be fully elucidated, accumulating evidence points to the important roles of glutathione and glutathione-dependent proteins in phytohormone signaling and defense against biotic stress (Noctor et al. 2011).

Oxidative Stress and Nutrition

The age we live in can be defined as a period in which the natural balance is deteriorating day by day, climate changes are increasingly felt, and perhaps as a reflection of these, increases in reactive oxygen species (ROS) are seen. Excess ROS has been associated with numerous chronic diseases, including asthma, diabetes, aging, cardiovascular disease, and neurodegenerative disease. The main challenge is the mechanic distinction between the toxic effects of oxidative stress and endogenous ROS functions, which occur at much lower concentrations (Kim et al. 2013). Similar to the relationship between ROS and diseases, diet and nutrition are known to play a key role in both pathogenesis and treatment in many chronic diseases (Fig. 3). For example, a strong correlation has been observed between inflammatory bowel diseases and dietary habits. It can manifest as food refusal or low intake of food due to nutritional disorders, behavioral problems, or underlying organic conditions. This situation mostly concerns infants and children under 6 years old; however, nutritional problems may also occur later in life (Rybak 2015). In one study, different dietary approaches to improve patients' symptoms were evaluated, and it was emphasized that useful guidelines should be defined to fully understand the effect of nutrition on the progression of diseases and that more studies are needed on this subject (Corsello et al. 2020). Another of the different approaches to nutrition is intermittent fasting (IF) (Fig. 4). IF is a term used to consume storage calories and to eat fewer calories by fasting for 12 h in repetitive periods, applied for days (Anton et al. 2018). Additionally, the increasing popularity of probiotics and prebiotics in nutritional applications cannot be ignored. Benefits of regular consumption of probiotics or prebiotics include improved immune function, improved colonic integrity, reduced incidence and duration of intestinal infections, downregulated allergic response, and improved digestion and elimination (Douglas and Sanders 2008). The prebiotic effect has been shown to be related to the modulation of biomarkers and the activity(s) of the immune system. The use of certain food products with a prebiotic effect has been tested in clinical trials to improve the clinical activity and well-being of patients. Numerous experimental studies have

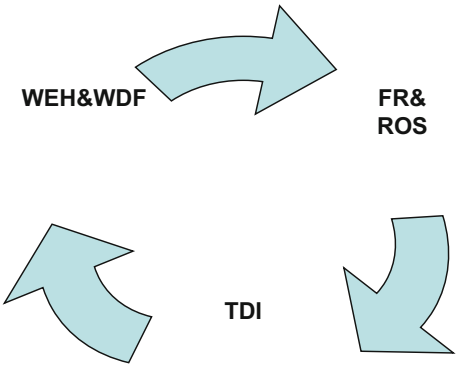

Fig. 3 The relationship between free radicals, thiol-disulfide imbalance, and diseases. *FR&ROS* free radicals and reactive oxygen species, *WEH&WDF* wrong eating habits and wrong diet preference, *TDI* thiol-disulfide imbalance

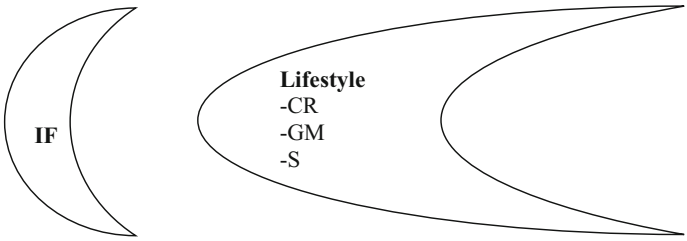

Fig. 4 Lifestyle change suggestions for intermittent fasting. *IF* intermittent fasting, *L* lifestyle, *CR* circadian rhythm, *GM* gut microbiome, *S* sleep

reported a reduction in the incidence of tumors and cancers after feeding certain food products with a prebiotic effect. Recent data from both experimental models and human studies support the beneficial effects of certain food products with prebiotic properties on energy homeostasis, satiety regulation, and body weight gain. These studies support the hypothesis that gut microbiota composition may contribute to modulate associated metabolic processes, particularly obesity and type 2 diabetes mellitus (Roberfroid et al. 2010).

Antioxidant Nutrition Priorities

The effects of enteral supplementation containing antioxidants after gastrointestinal surgery on blood antioxidant levels and oxidative stress indicators have been discussed previously (van Stijn et al. 2008). In the study, antioxidant enteral supplementation showed no adverse effects and was well tolerated. A decrease in circulating antioxidant parameters was observed after surgery. Lipopolysaccharide-binding protein (LBP) levels dropped faster in the antioxidant group after surgery. The rapid decrease in LBP levels in the antioxidant group suggested a possible protective effect on intestinal wall integrity (van Stijn et al. 2008). Malnutrition is cited as the leading cause of poor health in a comprehensive review of dietary priorities. It is recommended to focus on general dietary patterns instead of just counting calories, recognizing the effects of different foods on long-term weight regulation, and implementing new strategies for a lifestyle change. Evidence-based dietary priorities include increased fruit, non-starchy vegetables, nuts, legumes, fish, vegetable oils, yogurt, and minimally processed whole grains. On the other hand, red meat, processed meats, and refined grains, starch, added sugars, salt, and trans fats should be consumed less. More evidence-based studies are needed for other popular examples, such as organic or non-genetically modified product preferences (Mozaffarian 2016). An experimental study using an antioxidant-fortified diet was conducted in rats with type 2 diabetes mellitus and obesity. In this study examining the expression of nitric oxide synthase (NOS) in the kidneys, diabetic nephropathy (DN) in rats was emphasized. The development of DN has been associated with decreased renal nitric oxide production and increased oxidative stress (Slyvka et al. 2011).

It is also recommended to avoid smoking and alcohol consumption in antioxidant nutrition. In a meta-analysis, it was emphasized that there are significant morbidity and early mortality due to alcohol use, but this problem is largely undertreated. It was found that the medical drugs used were significantly underused (Jonas et al. 2014). In a study on the thiol-disulfide balance, patients with acute pancreatitis who came to the emergency department with the complaint of abdominal pain were examined (Ercan Haydar et al. 2020). Alcohol use was also determined in the study. While the blood thiol levels of the patients were low, disulfide levels were found to be significantly higher (Ercan Haydar et al. 2020). Dynamic thiol-disulfide homeostasis and the effect of smoking on homeostasis parameters were investigated in a study in patients with psoriasis. In the study, native thiol and total thiol levels were found to be significantly higher in patients than in the control group. In the study, when patients were divided into smokers and non-smokers, natural thiol and total thiol levels were found to be significantly higher in smokers compared to controls (Emre et al. 2017).

Healthy and Natural Nutrition

People's current dietary understanding of healthy nutrition is still inadequate. It is also very difficult to reach organic and natural products without additives in food preferences. On the other hand, even providing this is not enough for healthy nutrition because eating habits come to the fore as well. In addition to the characteristics of the food consumed, the amount and frequency of meals are also substantial. Intermittent fasting (IF), which has been researched in recent years in order to eat healthily, was examined in a review (Patterson and Sears 2017) (Fig. 4). This study, which summarizes the evidence on the health benefits of intermittent fasting through physiological mechanisms, was conducted on MEDLINE. It has been determined that fasting regimens can provide weight loss and thus have positive effects on metabolic health. In addition, it appears to support the hypothesis that eating habits that reduce or eliminate nighttime eating and extend nighttime fasting intervals can result in sustained improvements in human health. Intermittent fasting regimens are hypothesized to mediate metabolic regulation through effects on circadian biology, gut microbiome, and lifestyle behaviors such as sleep. If proven effective, these dietary regimens offer promising non-pharmacological approaches to improving health at the population level, with many public health benefits. In addition, it should be noted that Ramadan fasting (RF) is a form of IF practiced by millions of adult Muslims worldwide for 1 month each year (Lessan and Ali 2019). Avoiding additives in the preferences of the foods we eat, especially fructose (Hannou et al. 2018), poses a microplastic global risk in recent years (Sharma and Chatterjee 2017). When all these negativities are considered, antioxidant food preferences increase the importance of healthy nutrition a little more. In one study, antioxidant food preferences and skin aging were examined from a nutritional perspective (Cao et al. 2020). As it is known, the skin is the primary barrier that protects the body against external factors. Skin aging is a complex biological process

affected by internal and external factors. People need to reconsider their original lifestyle, as well as healthy dietary choices, which are necessary to improve skin aging.

Dietary Preferences and Oxidative Stress

Diet is a complex process associated with metabolism because energy balance and cellular stress response affect the process. Nutrition is also one of the main elements of a healthy life (Fig. 5). On the other hand, strengthening the immune system through nutrition and reducing oxidative stress are among the things to be considered in the current COVID-19 crisis (Iddir et al. 2020). As it is known, the coronavirus disease (COVID-19) in 2019 was declared as a global pandemic by the World Health Organization. Optimal immune response to viral diseases depends on an adequate diet and nutrition to keep infection at bay. For example, adequate protein intake is crucial for optimal antibody production. Low micronutrient status, such as vitamin A or zinc, has been associated with an increased risk of infection. Malnutrition is often associated with inflammation and oxidative stress, which can affect the immune system. Dietary components with particularly high anti-inflammatory and antioxidant capacity include vitamin C, vitamin E, and phytochemicals such as carotenoids and polyphenols. Many of these can interact with transcription factors such as NF-kB and Nrf-2, which are related to anti-inflammatory and antioxidant effects, respectively. Especially vitamin D, cell entry receptors (angiotensin-converting enzyme 2) can disrupt viral cellular infection by interacting with ACE2. Dietary fiber fermented into short-chain fatty acids by the gut microbiota has also been shown to produce anti-inflammatory effects. Inflammation and oxidative stress can be effectively reduced by optimal consumption of relevant nutrients to strengthen the immune system during the COVID-19 crisis (Iddir et al. 2020). Humans consume diets for survival, but the genetic pathways of gut microflora genetics, enzymatic polymorphism, and the role of plant-derived foods require deeper consideration. A key issue addressed by dietitians relates to the role played by reactive species (Bjorklund and Chirumbolo 2017). An imbalance between antioxidant defense and pro-oxidant load due to under- or over-nutrition can induce oxidative stress. Various foods and consumption patterns have been associated with various cancers, and

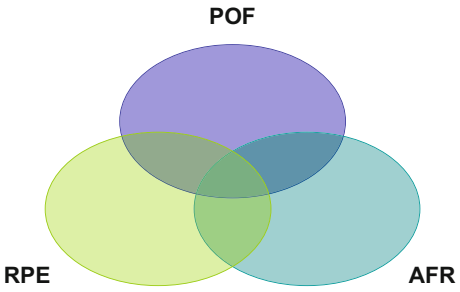

Fig. 5 Three important criteria for a healthy life. *POF* preferring organic food in the diet, *AFR* avoiding free radicals, *RPE* regular physical exercise

approximately 30–35% of cancer cases are associated with over-nutrition or malnutrition. However, there are several conflicting studies on the relationship between diet and cancer risk that await clarification. Oxidative stress is a physiological state in which high levels of reactive oxygen species (ROS) and free radicals are produced (Saha et al. 2017). On the other hand, free radicals are seriously blamed in diseases related to oxidative stress. In some studies, free radicals are regarded as the culprit of some diseases such as cancer and Alzheimer's disease. Increased generation of reactive oxygen species (ROS) in cancer cells from oncogenic signaling or metabolic disturbances leads to the upregulation of cellular antioxidant capacity to keep ROS levels below a toxic threshold (Poprac et al. 2017). At the same time, oxidative stress is a crucial factor for cancer progression and treatment (Tomasello et al. 2016).

Applications to Prognosis and Other Diseases or Conditions

Applications to Prognosis

We have shown that the thiol-disulfide balance can be a biomarker in the diagnosis and prognosis of many diseases in the studies we conducted in the laboratory of our hospital (Table 1). From this point of view, thiol-disulfide balance biomarkers can be used clinically to study prognosis in a wide variety of diseases. Indeed, other studies support this view. For example, it was concluded that thiol-disulfide imbalance due to oxidative stress in early-stage endometrial cancer might contribute to the etiopathogenesis of endometrial cancer (Sezgin et al. 2020).

Again, higher thiol levels in serum may be responsible for the increased proliferation of seborrheic dermatitis lesions (Emre et al. 2020). It has been determined that thiol oxidation is increased in type 1 diabetes mellitus (T1DM) patients compared to the control group (Ates et al. 2016a). The presence of chronic inflammation in these patients also coincides with the disruption of the thiol-disulfide balance and is a biomarker for increased oxidation (Ates et al. 2016a). There are important data that thiol-disulfide homeostasis can be preferred as a new oxidative stress mediator in acute appendicitis (AA) (Ozyazici et al. 2016). As a matter of fact, it has been stated that thiol-disulfide homeostasis will be a useful biomarker in children with AA (Elmas et al. 2020).

Applications to Other Diseases or Conditions

Many proteins found on cell surfaces and extracellular fluids contain cysteine and methionine residues that are subject to oxidation. These proteins respond to changes in the extracellular thiol-disulfide redox environment. Changes in the activity of these proteins can alter the ability of organs to function normally and affect processes such as nutrient absorption, secretory function, nerve conduction, and susceptibility to toxic substances. In addition, extracellular redox can regulate tissue homeostasis through effects on cell proliferation, differentiation, apoptosis, and immune function.

Consequently, extracellular redox can have significant effects on health status and disease states and thus may be a target for nutritional interventions (Moriarty-Craige and Jones 2004). Evidence from in vitro SARS-CoV-2 infection studies of thiol-based drugs to combat COVID-19, which has affected the whole world, is promising. The findings reveal that thiol balance is also important in terms of treatment (Khanna et al. 2020). Diseases include, for example, familial hypercholesterolemia. In the study in which 51 patients diagnosed with familial hypercholesterolemia and 81 healthy individuals were evaluated, it was found that the thiol-disulfide balance was impaired in favor of disulfide (Şimşek et al. 2018). It was determined that atopic dermatitis with increased oxidative stress might be associated with a new oxidative stress marker: thiol-disulfide balance (Karacan et al. 2020). It was stated that the results of dynamic thiol-disulfide balance in the patient group may shed light on the etiopathogenesis of atopic dermatitis and may be useful in the development of new treatment methods (Karacan et al. 2020). A similar result was obtained in the evaluation of thiol-disulfide homeostasis in patients with pityriasis rosea (Yüksel and Ülfer 2019). In a study on psoriasis, another skin disease, the etiopathogenesis of which has not yet been clarified, it was found that the thiol-disulfide balance shifted toward the thiol direction, especially in smokers. It was stated that this situation might be an important clue in the pathogenesis of psoriasis (Emre et al. 2017). Recent studies have shown that oxidative stress is one of the molecular changes underlying the pathogenesis of Alzheimer's disease. In this study, dynamic thiol-disulfide homeostasis in patients with Alzheimer's disease was investigated using a new method (Gumusyayla et al. 2016b). Oxidative stress is also accepted as one of the molecular changes in the pathogenesis of migraines. Dynamic thiol-disulfide homeostasis was investigated as a new oxidative stress parameter in migraine patients. Total and natural thiol levels of migraine patients participating in the study were found to be significantly higher than the total and natural thiol levels of healthy individuals (Gumusyayla et al. 2016a). It has been stated that these newly developed tests can be used as oxidative stress biomarkers in the pathogenesis of Alzheimer's disease (Gündüztepe et al. 2020).

Key Facts of Thiol-Disulfide Homeostasis as an Oxidative Stress Indicator: Applications to Nutrition

Thiol-disulfide homeostasis: Oxidative stress conditions can lead to reversible mixed disulfide formation. The disulfide bonds formed can be reduced back to thiol groups; as a result, the dynamic thiol-disulfide balance is preserved.

Free radicals and nutrition: The imbalance between ROS production and homeostasis, which has specific consequences for cell physiology, has been termed "oxidative stress." Much of the literature supports the idea that dietary antioxidants play an essential role in the prevention of many human diseases.

Antioxidant dietary priorities: Evidence-based dietary priorities include increased fruit, non-starchy vegetables, nuts, legumes, fish, vegetable oils, yogurt, and minimally processed whole grains. On the other hand, red meat, processed

meats, and refined grains, starch, added sugars, salt, and trans fats should be consumed less.

Healthy and natural nutrition: It is crucial to reach organic and natural products without additives in food preferences. On the other hand, the amount of food consumed and the frequency of meals are also essential for a healthy diet.

Summary Points

- Thiol-disulfide homeostasis can be considered as an indicator of oxidative stress.
- Many chronic, systemic diseases are associated with thiol-disulfide balance.
- Choosing an antioxidant diet is important for a healthy diet.
- We should stay away from the consumption of food that is exposed to free radicals.
- In order to eat healthy, we should prefer natural and organic products.
- Many chronic diseases are related to a combination of nutrition and oxidative stress.

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Oxidative Stress Biomarkers in Exercise

39

Intake of Supplements

Yıldırım Kayacan and Hayrullah Yazar

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Abstract

An increase in the amount of free radicals resulting from exercise has been reported in various studies on humans and experimental animals to date. Vigorous exercise increases the metabolic rate, resulting in an increase in oxygen consumption and an increase in free radical production. In cases where there is no or

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insufficient antioxidant mechanism that removes free radicals from the living organism, the damage to the body by oxidative stress cannot be prevented. It is common practice to use dietary supplements to increase the benefits of exercise, reduce biological problems, and improve performance. Consumption of foods rich in antioxidants in the diet during or after exercise plays a key role in reducing this damage. Various nutritional strategies, especially in athletes, are being studied by researchers to reduce oxidative stress at the cellular level. In this part, indicators of exercise-induced oxidative stress and the effect of supplementation on this mechanism are presented.

Keywords

Oxidative stress · Exercise · Redox · Supplements · Nutrition · Free radicals

Abbreviations

AA	Ascorbic acid
BDNF	Neurotrophic factor
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
GHS	Glutathione
GPX	Glutathione peroxidase
NAC	*N*-Acetyl-cysteine
OS	Oxidative stress
OX-LDL	Oxidized low-density lipoprotein
PC	Protein carbonyls
PLP	Pyridoxal 5′-phosphate
ROS	Reactive oxygen species
-SH	Sulfhydryl
SOD	Superoxide dismutase
SS-	Disulfide
VA	Vitamin A
VB6	Vitamin B6

Introduction

Oxidative Stress and Redox

The concept of oxidative stress was first used by Helmut Sies (1985) in 1985 in the first chapter of his book "Oxidative stress: introductory remarks" for the studies in redox biology and medicine. Then, it attracted great attention from many researchers, and this concept was examined in different fields of study (exercise, medical, nutrition, etc.). Over time, redox biology/physiology as a research area has become a parameter used in a wide range of disciplines, starting from chemistry and radiation biology, to biochemistry and cell physiology, to general biology and other

branches of medicine. The first of these concepts is "oxidative" (reducing), which is expressed as an important concept of aerobic mechanism, and the second is "stress," as a biological response.

The idea that oxidation-reduction reactions described as redox in living cells are used in basic redox regulation processes collectively called "redox signaling" and "redox control" was admitted in the scientific world and gained an important place. In the process, the concept of oxidative stress was also updated by Sies and Jones in 2007 to include the role of redox signaling (Sies and Jones 2007) and was recently conceptualized as the redox code (Jones and Sies 2015).

Oxidative stress has been defined as an imbalance between oxidant and antioxidant levels that suppresses the antioxidant defenses of oxidant production, often leading to the oxidation of lipids, proteins, DNA, and other molecules in ways that impair cellular function, and as a condition that leads to disruption of redox signalling and control and/or molecular damage. The main idea underlying this global concept is the maintenance of a redox balance, also called redox homeostasis, in living biological systems (Fig. 1).

Specifically, oxidative stress can be caused by different conditions. For example, it may occur through increased activity of radical-producing enzymes; activation of cyclooxygenases, phospholipases, lipoxygenases, and phagocytes; the release of its proteins through the destruction of iron-containing proteins; and through further leakage of superoxide radicals through disruption of the electron transport system and suppressed antioxidant protection.

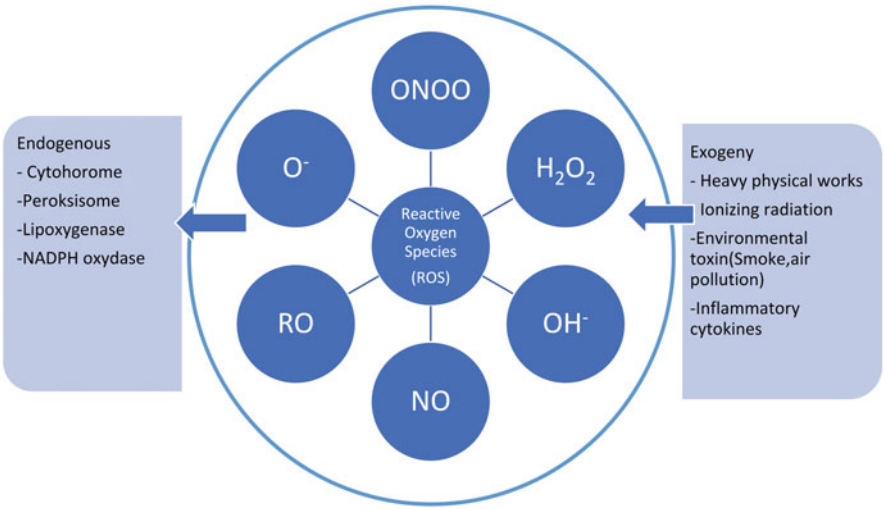

Fig. 1 Endogenous and exogenous oxidant sources. (ONOO, peroxynitrite; O, oxygen; NO, nitric oxide; OH, hydroxyl radical; RO, alkoxyl radical; H_2O_2, hydrogen peroxide). In addition to normal cellular activities in metabolism, oxidants can be produced through exposure to a wide variety of environmental (air pollution, cigarette smoke, certain food types, etc.) and physiological (exercise, heavy workload, physical and mental stress, etc.) factors from endogenous or exogenous sources

Oxidative Stress in Exercise

The subject of exercise-induced oxidative stress has attracted great attention in recent years, with more than 300 original studies published since Dillard et al. It was first reported in 1978 that lipid peroxidation increased after 60 min of cycling exercise (Dillard et al. 1978). In fact, physical activity/exercise is a recommended method as an anti-inflammatory treatment method. A regular exercise is a natural form of protection against chronic inflammatory diseases by incorporating anti-inflammatory cytokines into the circulation. However, acute physical activities can initiate/accelerate a series of inflammatory processes depending on their intensity and duration. The important parameter we encounter here is how much energy is used during the exercise process. Intensive and vigorous activities that require more energy cause significant release of proinflammatory cytokines and free radicals from leukocytes and muscle and tissue damage. In addition, oxygen consumption increases during long-term exercise, and reactive oxygen species (ROS) production is accelerated through the electron transport chain. There are many studies in the literature on oxidative stress caused by exercise. ROS cause oxidative damage to protein, carbohydrate, and lipid molecules (Fittipaldi et al. 2014). Lipid peroxidation caused by ROS (Su et al. 2019) and carbohydrate oxidation products also cause modifications in the amino acid content of proteins and cause an increase in plasma protein carbonyl content (Singh et al. 2015). ROS that occur suddenly in skeletal muscle can cause damage to skeletal muscle cells. Long-term and strenuous exercise causes damage to the sarcoplasmic membranes of skeletal and cardiac muscle cells, deterioration of muscle contractility and myofibril structure, and changes in some biochemical parameters, including blood urea, creatine kinase, and lactate dehydrogenase activity (Salo et al. 1991). Exercises cause oxidative stress of varying intensity from each other depending on parameters such as the form, intensity of the exercise, and the muscle group it works (Kayacan et al. 2019b) (Fig. 2).

Free radical and ROS production in exercise occurs in several ways (Kayacan et al. 2019a, b). The first is the loss of electrons in the mitochondria, and the other is the lack of circulation, blood, and oxygen during high exercises (Zorov et al. 2014).

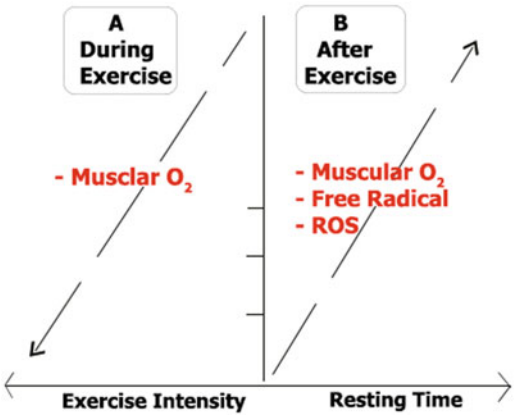

Fig. 2 Skeletal muscle during and immediately after exercise. Oxygen is depleted in the muscles when the maximum oxygen consumption level (VO_2Max) is reached, especially during intensive physical activity. Potentially reactive oxygen species and free radicals emerge as muscle oxygen stores rapidly fill up after exercise

In general, it has been found that oxygen metabolism in mitochondria is associated with free radical production. It has been determined that 95–99% of the consumed oxygen is converted to water by the cytochrome oxidase system in the mitochondria, and the remainder (1–5%) leaks from the mitochondria during oxygen consumption and forms the superoxide radical (Ott et al. 2007). The increase in oxygen needed with exercise causes the proliferation of free radicals.

It is emphasized that the increase in free radical production during long-term submaximal aerobic exercise is mainly due to the great increase in oxygen consumption. Over the last 30 years, our knowledge of the biological effects of exercise-induced oxidative stress has grown rapidly. Indeed, it is known that high levels of free radicals can damage cellular components, and low/intermediate oxidants play regulatory roles such as control of gene expression, regulation of cell signaling pathways, and modulation of muscle strength formation in skeletal muscles (Kayacan et al. 2019a).

Antioxidants come into play to prevent oxidative stress damage that will occur in our bodies. They give their excess electrons or hydrogens to free radicals. In order to prevent the damage caused by oxidative stress, there must be sufficient antioxidants in the environment, that is, in the body (Valko et al. 2006).

Applications to Prognosis

Intake of Supplements in Exercise/Physical Activation and Redox

Nutritional supplements used to alter athlete performance and body composition are widely used around the world. Those who use these products the most are especially professional athletes. The desire to improve the quality of performance and bring it to the top has made the use of these substances more attractive. The American College of Sports Medicine, Dietitians of Canada, and the American Dietetic Association state that there are people who exclude certain food groups that only restrict energy intake and cause rapid weight loss from their diets or consume high amounts of them (Rodriguez et al. 2009). Some supplements contain potentially toxic ingredients in an overdose, while others generally do not contain a substance harmful to the body. Supplements are often advertised and commercialized for specific purposes. Basic marketing strategies include factors such as increasing performance and muscle mass, weight control, resistance to diseases, increasing alertness and mental activities, and reducing stress. They are marketed and bought thinking the potential to produce miraculous and magical results. OS from exercise can be turned into an advantage in endurance athletes by being neutralized by the response of a much higher antioxidant system. However, the desire to maximize sustainable physical activity and athletic performance require additional resources in order to quickly and healthily eliminate the damage caused by exercise. In this context, supplements and active ingredients obtained from plants have been preferred as a widely used method, especially for athletes. The following table provides some examples of research examining nutritional protocols to reduce exercise-induced oxidative stress (Table 1).

Some of the studies on supplementation and nutritional strategies used in studies evaluating physical activity and oxidative stress studies together and for the

Table 1 Examples of research on exercise and nutrition protocols related to oxidative stress. In order to reduce or eliminate oxidative stress caused by exercise, some nutrition-oriented research protocols, different exercises, and subject and duration applications continue to be widely investigated

Author(s)	Used supplement	Exercise type	Participant type	Time
Toldy et al. (2005)	*Urtica dioica*	Swimming	Rat	6 weeks
Silva et al. (2011)	Taurine	Downhill run (eccentric exercise)	Rat	15 days
Ammar et al. (2017)	Pomegranate juice	Weightlifting	Athlete	Acute study
Petiz et al. (2017)	Vitamin A	Swimming	Rat	8 weeks
Basham et al. (2020)	Curcumin	Specific muscle damage exercise	Healthy male	28 days
Darband et al. (2020)	L-Arginine	Treadmill exercise	Rat	12 weeks
Le Garf et al. (2021)	Alpha-lipoic acid+diet	Treadmill and water exercise	Rat and women	8 weeks
Chupel et al. (2021)	Taurine	Specific combined exercise	Older women	12 weeks
Yimcharoen et al. (2019)	Ascorbic acid	Cycling (Akut)	Healthy women	Acute study

elimination of oxidative damage are explained in Table 1. The common feature of these substances is that they have antioxidant-containing structures. The first human study on the subject was the determination of the effect of the *N*-acetyl-cysteine (NAC) infusion with a cysteine donor that increases GHS, by Reid et al. (1994). With this study, it was determined that reactive oxygen species and free radicals affect muscle fatigue; this situation can be reversed with antioxidant support, and physical performance will be increased in systemic endurance training.

Many studies in the literature stated that antioxidant enzymes also increase with the increase of physical exercise and oxidative stress. This increase may not be physiologically proportional to the needs created by the increase in prooxidant events. Therefore, the need for vitamins, as an antioxidant method, may be affected. Of course, factors such as duration and intensity of exercise, age, gender, and overall physical capacity will be affected. In studies, it has been observed that the basic nutrients used in reducing/eliminating oxidative stress caused by exercise are concentrated in vitamins (Table 2).

Nutritional Oxidative Stress Biomarkers in Exercise

Protein Carbonyl Groups

Protein carbonyl (PC) groups have some advantages over the measurement of other oxidative stress parameters due to the stability and relative early formation of carbonylated proteins (Ceci et al. 2014). Analyses to determine these parameters

Table 2 Enzymatic and non-enzymatic antioxidant types in aerobic organisms. Aerobic organisms present enzymatic and non-enzymatic antioxidants. However, vitamins, as a nutritional profile, are among the important sources of antioxidants, especially in exercise

Enzymatic antioxidants	Non-enzymatic antioxidants	Antioxidant vitamins
Superoxide dismutase (SOD)	Albumin Anthocyanidins Bilirubin Flavonols Flavones Isoflavones Flavanones Flavonols Folic acid Gallic acid Glutathione (GSH) Lipoic acid Resveratrol Ubiquinol, uric acid Phenolic acids Thioredoxin (TRX) Ubiquinone	Vitamin E α-Tocopherol
Glutathione peroxidase (GPx)		Vitamin A Astaxanthin α-Carotene β-Carotene β-Cryptoxanthin Lutein Lycopene Zeaxanthin
Catalase (CAT)		Vitamin C Ascorbic acid
Glutathione reductase (GR)		Vitamin B6 Vitamin B9 Folic acid
		Vitamin K Phylloquinone Menaquinone

include derivatization of the carbonyl group leading to the formation of a stable dinitrophenyl (DNP) hydrazone product with 2,4-dinitrophenylhydrazine (DNPH). This method can be detected by means such as ELISA or Western blot (Dalle-Donne et al. 2003). This method is also used in the detection and interpretation of exercise-induced supplement use and changes in redox balance (Pappas et al. 2021; Yada et al. 2020). However, findings regarding the amount of PC detected in plasma in response to nutrition with exercise are contradictory. It is thought that many factors such as the intensity, duration of the exercise, and the participant profile are responsible for detecting these contradictions (Wadley et al. 2016).

Oxidized Low-Density Lipoprotein

Measurement of oxidized low-density lipoprotein as a biomarker of oxidative stress has been associated with oxidative modification of atherosclerosis. It is also used as an important marker for obesity and cardiovascular diseases. In general, these types of diseases are common in the elderly population, with decreased physical activity and increased body weight; exercise studies have also focused in this direction. In a study, it was determined that there is a relationship between plasma oxidized LDL level and physical activity in the elderly (Galan et al. 2006). In another study, it was reported that there is a relationship between circulating OLDL concentration and body mass index, lumbar fat thickness, and general fitness level (Bachi et al. 2019).

Thiol-Disulfide Homeostasis

Thiol groups are organic, essential, and strong antioxidant molecules containing sulfhydryl (-SH) group that defend the organism against the destructive effects of oxidative stress damage. Thiol groups in cysteine, homocysteine, glutathione, albumin, and other proteins are the primary target of ROS (Benedikter et al. 2018). Thiols can undergo oxidation reactions with oxidants and disulfide bonds. The disulfide bond is a covalent bond, also called a disulfide (-SS-) bridge. Under oxidative stress conditions, cysteine residues can recycle and lead to the conversion of mixed disulfides into protein disulfide and low molecular mass thiol groups. The form of disulfide bonds can be reduced back to thiol groups, thus maintaining dynamic thiol-disulfide homeostasis (Erel and Neselioglu 2014). Thiols can enter into an oxidation reaction via oxidants to form disulfide. When oxidative stress increases, oxidation of cysteine residues can lead to the formation of mixed disulfides of molecular weights between the thiols and the protein thiol group. However, this formation is reversible in any case. Disulfide bonds can also be reduced in thiol groups, resulting in thiol/disulfide homeostasis. Dynamic thiol/disulfide homeostasis plays an important role in cell signaling mechanisms, transcription factors, enzymatic regulation, activations, apoptosis and signal transduction, antioxidant protection, and detoxification.

The first studies on thiol-disulfide homeostasis as an oxidative stress parameter in exercise were studies on L-tyrosine, an antioxidant amino acid with amphiphilic properties due to its polar and apolar groups, and 5-hydroxytryptophan, a serotonin precursor (Kayacan et al. 2018). This parameter, which has a high level of validity and reliability, is seen as a biomarker that can also be used in studies related to exercise and nutrition.

Oxidative Stress Caused by Exercise and Vitamin Diet

Vitamins C and E

Ascorbic acid and vitamin E are widely used by athletes to increase antioxidant capacity and suppress excessive reactive oxygen species during/after exercise, although the results are controversial. In studies, it has been stated that these vitamins reduce oxidative stress, although they do not have an effect on muscle damage, muscle strength, agility, and performance, especially in acute applications (de Oliveira et al. 2019). In different studies, it was determined that vitamin C taken after exercise reduces lipid peroxidation and interleukin-6, which are inflammatory markers (Poulab et al. 2015; Davison and Gleeson 2007). However, another study reported that these markers were not affected, and the results are still controversial (Urso and Clarkson 2003). In a non-exercise study, its effect on cognitive function, serum level of brain-derived neurotrophic factor (BDNF), and the activity of antioxidant enzymes in the brain were investigated in ovariectomized mice based on the antioxidant property of ascorbic acid (AA). As a result, AA given at different doses prevented the detrimental effects of ovariectomy on learning memory and working

memory in the brain. Serum BDNF, SOD, and GPx activity were also increased (Delrobaei et al. 2019). In a study conducted in rats, the effect of exercise and vitamin E on reducing oxidative stress caused by d-galactose administration was investigated. As a result, it was determined that 4-week free walking exercise and vitamin E supplementation significantly reduced the level of advanced glycation end products in the blood, both as intracellular ROS and serum aging index. Compared with the exogenous lipophilic vitamins found in the body, the concentration of vitamin E, which is mostly found in the cell membrane, is very high. Therefore, vitamin E is thought to have an active role in the regulation of redox interactions in the body.

Vitamin A

Vitamin A (VA) is a fat-soluble vitamin obtained from different compounds, indicated to be involved in the redox process. It plays a role in the prevention of lipid peroxidation with the regulation of long chains of conjugated double bonds common to all retinoids (Ozhogina and Kasaikina 1995). Reviews of this vitamin's effects on exercise-induced oxidative stress often mention VA or its precursor β-carotene as a potential antioxidant molecule (Sumida et al. 1997; Vincent et al. 2006). However, in research on its effects on exercise, the in vivo mechanisms have remained unclear. In a study, it was determined that VA supplementation did not show an antioxidant effect in rats exercising but decreased the benefits of exercise in skeletal muscle, upregulation of superoxide dismutase-2, anti-inflammatory interleukin-10, and heat-shock protein 70 expression (Petiz et al. 2017a). In parallel with this finding, it was determined that it caused liver lipid peroxidation and protein damage in rats exercising and prevented the increase in HSP70 expression obtained with exercise. Only higher levels of antioxidant enzyme activity were detected in the exercising group, and VA inhibited this adaptation (Petiz et al. 2017b). In one study, the antioxidant activity of all-trans-retinol was investigated based on characteristic thermochemical properties. It has been determined that retinol can act as an effective antioxidant as well as a prooxidant in generating reactive hydroxyl radicals (Dao et al. 2017). In this context, it is thought that the ability of VA to be an antioxidant that can be used in the ROS production process resulting from exercise is weak.

Vitamins B6 and B9

Vitamin B6 (VB6) is a water-soluble vitamin readily found in animal and plant foods in the forms of pyridoxal, pyridoxine, and pyridoxamine (Selam et al. 2015). After digestion and absorption, the liver converts VB6 into the biologically active form, pyridoxal 5′-phosphate (PLP). PLP then binds with albumin and corresponds to approximately 60% of circulating vitamin B6 (Parra et al. 2018). Importantly, PLP serves as a cofactor for numerous reactions related to macronutrient metabolism and immune response (Yoshii et al. 2019). It has been reported that in vitamin B6

deficiency, it is associated with some defense mechanisms against lipid peroxidation in experimental animals, especially in tissues. However, the content of this vitamin increases lipid peroxidation and significantly stimulates the activity of the glutathione-dependent enzyme (Ravichandran and Selvam 1991). In one study, rats were given a vitamin B6 deficiency diet with exercise. As a result, it has been determined that oxidative stress caused by exercise may become more severe in B6 deficiency (Choi and Cho 2009). These findings were determined in the same direction in rats performing swimming exercises (Benderitter et al. 1996). Vitamin B6 has been found to restore neutrophil infiltration, oxidative markers in the liver, and catalase activity levels in the lungs of septic animals and show anti-inflammatory and antioxidant effects in peripheral organs after sepsis (Giustina et al. 2019). Folic acid is a water-soluble micronutrient important for growth, reproduction, and bodily functions. It is a cofactor in many metabolic reactions such as DNA-RNA and mitochondrial protein synthesis and production of *S*-adenosylmethionine with its reduced derivatives (Brocardo et al. 2010). In some studies, on the other hand, it has been reported that folic acid has antioxidant properties (Joshi et al. 2001; Dhitavat et al. 2005). However, it is seen that the research profile including exercise/sports performance and folic acid application in the literature is insufficient.

Mini-dictionary of Terms

- **Oxidative stress**: Oxidative stress reflects the imbalance between the systemic manifestation of reactive oxygen species and the ability of a biological system to readily detoxify reactive intermediates or repair the resulting damage.
- **Redox**: It is a kind of chain reactions chemically related to redox atoms.
- **ROS**: Reactive oxygen species are highly reactive chemicals composed of O_2.
- **Supplements**: It is the common name given to the nutrients generally used by athletes to increase performance or accelerate recovery.
- **Thiol-disulfide homeostasis**: A biomarker with high validity and reliability used in many areas in the determination of oxidative stress.

Key Facts of Oxidative Stress in Exercise and Nutrition

Exercise or physical activity produces oxidative stress, depending on its duration, type, or intensity.

The main indicator of oxidative stress in exercise is the amount of oxygen use.

Oxidative stress is influenced by factors such as gender, age, general health, and fitness level.

Some nutritional strategies have been developed to reduce/eliminate oxidative stress, which is an important problem in exercise, especially in performance athletes.

Vitamins continue to be widely researched in studies aimed at reducing oxidative stress in athletes.

Summary Points

- Different biomarkers are used to determine oxidative stress in exercise. In the determination of the biomarker used, the type and number of participants in the research and the exercise protocol are the main determinants.
- It is seen that vitamins are used extensively in studies on oxidative stress in athletes. However, the findings regarding some vitamins are quite contradictory.
- The main reasons for the differences in nutrition and oxidative stress findings in exercise include exercise type, intensity, duration, and participant profile.
- The first research on oxidative stress in exercise was carried out in 1978. Since then, research on the subject has continued with newly found biomarkers.
- Researchers continue to develop various protocols for nutritional strategies to increase athlete performance, accelerate recovery, and prevent injuries.

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Biological Indicators of Oxidative Stress [Malondialdehyde, Catalase, Glutathione Peroxidase, and Superoxide Dismutase] and Their Application in Nutrition

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Abstract

The imbalance between the oxidant and antioxidant systems leads to a biological condition defined as oxidative stress that contributes to the formation of reactive oxygen species, which are unstable molecules resulting from various processes in the body that can be highly harmful and cause a series of diseases. In this sense, the action of antioxidants becomes necessary, as they are responsible for inhibiting and reducing damage caused by free radicals in cells. However, it is possible to prevent the damage caused by exposure to pro-oxidant substances through the measurement of biological biomarkers, which are important in the evaluation of the redox state. In this chapter, we will discuss markers used to assess the capacity of free radical scavenging, such as catalase, glutathione peroxidase, and superoxide dismutase enzymes, in addition to malondialdehyde, a biomarker used to assess lipid peroxidation. Oxidative stress biomarkers have been studied in several pathologies and can help in the diagnosis and monitoring of the progression of these diseases.

Keywords

Oxidative stress · Antioxidants · Superoxide dismutase · Metalloproteins · Reactive oxygen species · Lipid peroxidation · Malondialdehyde · Biomarkers · Catalase · Glutathione peroxidase · Selenoproteins

Abbreviations

BMI	Body mass index
CAT	Catalase
COPD	Chronic obstructive pulmonary disease
Cu-Zn-SOD	Copper, zinc superoxide dismutase
CVD	Cardiovascular diseases
DM2	Type 2 diabetes mellitus
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
Fe-SOD	Iron superoxide dismutase
FMOC	9-fluorenylmethoxycarbonyl hydrazine
GPx	Glutathione peroxidase
GPx-1	Glutathione peroxidase 1
GPx-2	Glutathione peroxidase 2
GPx-3	Glutathione peroxidase 3
GPx-4	Glutathione peroxidase 4
GR	Glutathione reductase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HPLC	High-performance liquid chromatography

KCN	Potassium cyanide
LDL	Low-density lipoproteins
LP	Lipid peroxidation
M1dG	3-(2-deoxy-β-d-erythro-pentafuranosyl) pyrimido[1,2-α]purin-10 (3H)-one deoxyguanosine
MDA	Malondialdehyde
Mn-SOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
NU	Nitrite units
O_2•-	Superoxide anion
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SNP	Single-nucleotide polymorphism
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SOD3	Superoxide dismutase 3
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TXA2	Thromboxane A2
U/g Hb	Units per gram of hemoglobin

Introduction

Lifestyle components of most of the population have become a target of concern due to potential risks to human health, since several aspects related to them make the body more susceptible to oxidative stress due to the imbalance between the oxidant and antioxidant systems that results in the generation of reactive oxygen species (ROS) or free radicals (Kunst et al. 2014).

The production of ROS triggers different antioxidant defense mechanisms to control intracellular concentrations of reactive species and limit the damage caused by them (Vasconcelos et al. 2014). When the antioxidant defense system is unable to neutralize the action of ROS, whether due to a loss or deficiency in its mechanism, oxidative stress occurs. The imbalance promoted by this biological condition is associated with the emergence of numerous diseases. As a result, biological processes subject to oxidative stress can suffer various effects, including cytotoxicity, mutations, and chromosomal abnormalities (Vásquez et al. 2011) (Fig. 1).

In this context, the cells' antioxidant defense systems can be classified into enzymatic and nonenzymatic, and have the function of protecting cellular components and preserving the redox state of the cell (Dalvi et al. 2013). The effectiveness of the defense of these systems depends on the adequate consumption of micronutrients such as vitamins and minerals (nonenzymatic antioxidant defense system), and on the production and action of enzymes that metabolize free radicals (enzymatic system) such as catalase (CAT), superoxide dismutase (SOD), and glutathione

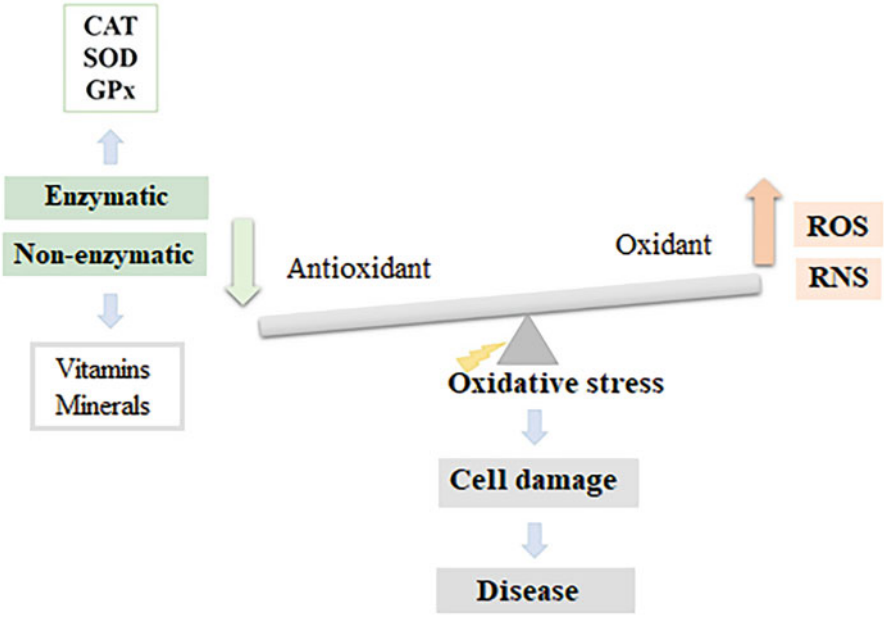

Fig. 1 Schematic representation of the oxidative stress process
Legend: Imbalance of oxidant and antioxidant agents, favoring pro-oxidants, characterizes the condition of oxidative stress responsible for cell damage and onset of diseases. The oxidant elements are reactive oxygen and nitrogen species and the antioxidant components are the enzymes catalase, superoxide dismutase, and glutathione peroxidase, in addition to nonenzymatic elements such as vitamins and minerals. Source: Prepared by the authors (2021)

peroxidase (GPx) which are also used as markers of oxidative stress (Vasconcelos et al. 2014).

It is possible to prematurely prevent the damage caused by exposure to pro-oxidant substances, which are harmful to the body, through biomonitoring. The latter makes it possible to identify risk factors for the development of various pathologies. The biological investigation of exposure to chemical agents is an analysis of substances or their metabolites through parameters called biological indicators or biomarkers (Muniz et al. 2008). One of the most studied biomarkers of oxidative stress in humans is malondialdehyde (MDA), an aldehyde formed from the lipid peroxidation reaction that provides information about the redox state (França et al. 2013).

Among the advantages of using biomarkers is the fact that they are simpler, have a reduced cost when compared to other parameters, and can be reevaluated in short periods of time (Aronson and Ferner 2017). Antioxidant evaluation methods are classified into two types: tests used to assess lipid peroxidation and tests used to verify free radical scavenging capacity (Alves et al. 2010).

Oxidative stress biomarkers have been studied in several diseases, including cardiovascular disease, diabetes, kidney disease, obesity, neurodegenerative

disorders, and cancer, among others. The evaluation of the redox state can help in the diagnosis and progression of these diseases; however, the existence of several markers with different determination methodologies makes it difficult to reproduce and compare the results (Marrocco et al. 2017).

Catalase (CAT)

Characterization and Historical Basis

Catalase is an enzyme that integrates the antioxidant defense system and is responsible for the dismutation of hydrogen peroxide (H_2O_2) in which it is converted to water and molecular oxygen (Scaglione et al. 2016). It is an enzyme that is widely found in almost all eukaryotic cells, especially in the peroxisomes and cytoplasm of these cells, providing cell protection against the toxic effects of H_2O_2 (Labios et al. 2009).

Historically, catalase was first described by Thénard (1818) in the discovery of hydrogen peroxide, as a substance with special activity that acted on the degradation of H_2O_2 in tissues. Later, in 1900, Loew named this substance as catalase, an enzyme with the ability to catalyze the reaction of conversion of hydrogen peroxide into water and oxygen (Loew 1900). This researcher discovered the existence of catalase in a variety of beings, in animal and plant tissues.

In the 1920s, Warburg and collaborators determined, through the use of cyanide for enzyme inhibition, that catalase has an active site that has an iron atom as an integral part (Warburg 1923). Years later, Stern (1937) demonstrated that the heme group present in catalase has the ability to react with other substances, especially cyanides, sulfides, and fluorides, and also that there is a ferric complex in the active site of this enzyme, which is the same protoporphyrin present in hemoglobin, in erythrocytes. In the same period, catalase was purified and crystallized for the first time from bovine tissue (Sumner and Dounce 1937).

Determination Methods

Catalases are classified, according to their physical-chemical, sequential, and structural characteristics, in three distinct forms: (1) typical catalases, identified in living beings that present aerobic respiration; (2) catalase-peroxidases, detected in fungi, archaebacteria, and bacteria; and (3) nonhematic catalases, exclusive to bacteria. The first two forms consist of enzymes that have the heme group, and the third is the class that contains manganese (Glorieux and Calderon 2017; Nandi et al. 2019).

The activity of the enzyme catalase can be determined in different biological samples, such as erythrocytes, serum, plasma, tissue homogenates, and even in vitro, through a variety of techniques that include: spectrophotometric methods (Beers and Sizer 1952; Aebi 1984; Hadwan 2018), colorimetric methods (Sinha 1972), catalytic polarographic currents (Rigo and Rotilio 1977), chemiluminescence (Mueller et al.

1997), gasometric methods (Siqueira et al. 1999), gel activity assay (Weydert and Cullen 2010), high-performance liquid chromatography (HPLC) (Böhmer et al. 2011), and flow injection analysis (Ukeda et al. 2004; Nashar 2012). Further, some studies also used specific kits to determine the activity of this enzyme (Usman et al. 2019; Verma et al. 2018). However, despite the different methodologies, ultraviolet spectrophotometric methods are often the most used to determine enzyme activity (Hadwan 2018), since they are simpler and cheaper techniques (Zezzi-Arruda and Poppi 2005).

Ultraviolet spectroscopy is based on measuring the ultraviolet absorption of H_2O_2 (Beers and Sizer 1952). According to Aebi (1984), the principle of the method is based on absorbance monitoring, in which catalase activity is measured following the degradation of hydrogen peroxide at 240 nm. Thus, the difference in absorbance over a period of time is proportional to a measure of enzyme activity.

Importance and Applications to Prognosis, Other Diseases, or Conditions

Catalase is classified as a first line of defense antioxidant that plays an important role in eliminating or preventing cellular generation of reactive oxygen species, quickly inactivating substances with a potential harmful effect on cells. Catalase has high efficiency and, in addition to rapid decomposition of hydrogen peroxide due to its peroxidase activity, it can also react with other molecules such as methanol, ethanol, formic acid, or phenols (Ighodaro and Akinloye 2018).

Catalase has numerous applications with wide scope, and is used in the chemical, pharmaceutical, food industry, environment, and medical and therapeutic areas (Sooch et al. 2014). In the latter two areas, its application is based on its relevant function in the elimination of hydrogen peroxide in human erythrocytes, as demonstrated in the experiment by Gaetani et al. (1994), in which catalase removed about half of the hydrogen peroxide produced in those cells.

Deficient or altered activity of catalase is related to neurological and metabolic disorders (Nandi et al. 2019). Decreased catalase activity has been reported in Parkinson's disease (Yuan et al. 2016), in type 2 diabetes mellitus (DM2) (Arpaci et al. 2020), in some types of cancers (Cobanoglu et al. 2010; Zińczuk et al. 2019), cardiovascular diseases, and chronic obstructive pulmonary disease (Casado et al. 1998) (Fig. 2).

In addition, catalase deficiency, considered a genetic disorder described as acatalasemia, was related to an increased risk of developing DM2 as a result of the increased susceptibility of pancreatic β cells to oxidative stress due to the overabundance of hydrogen peroxide resulting from the deficit of enzyme, preventing the performance of its critical protective function of these cells (Goth and Nagy 2012).

Catalase activity has also been investigated in relation to nutritional status. In this context, in a study that evaluated the activity of this enzyme, the single-nucleotide polymorphism (SNP) of the catalase gene (−844A/G) and its relationship with childhood obesity, catalase activity was significantly lower in children with obesity

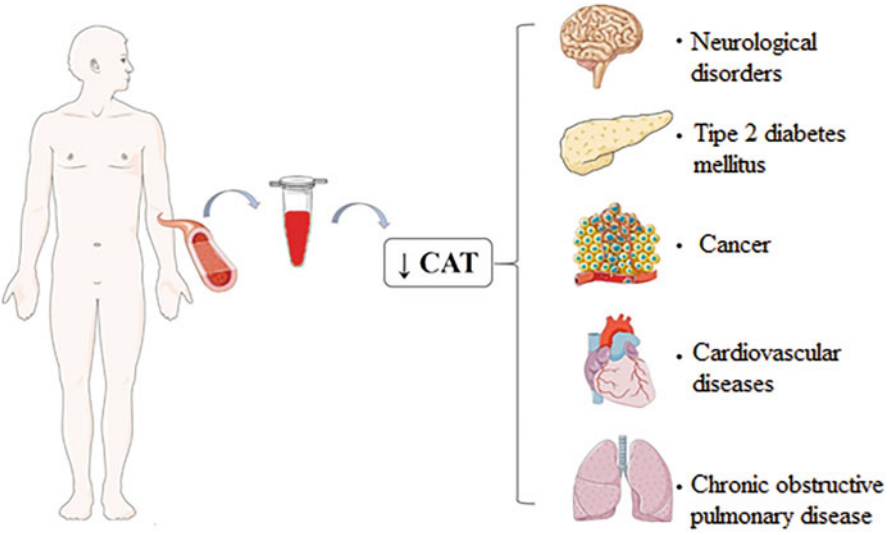

Fig. 2 Diseases related to reduced catalase enzyme activity
Legend: The figure demonstrates the pathologies associated with reduced activity of catalase; they are neurological disorders, DM2 – type 2 diabetes mellitus, cancer, CVD – cardiovascular diseases, and COPD – chronic obstructive pulmonary disease. SNIP from smart.server.com (2021) with permission.

and enzymatic activity was significantly correlated with obesity parameters and with insulin resistance and oxidative stress biomarkers. Furthermore, as for polymorphism, it was also associated with a higher risk of childhood obesity (Rupérez et al. 2013).

In another study that evaluated the activity of antioxidant enzymes in the body weight recovery process in anorexia nervosa, catalase activity significantly increased after improving dietary intake and weight recovery (Oliveras-López et al. 2015).

The evaluation of catalase activity for use in clinical diagnosis has presented limitations due to the existence of a variety of analytical methodologies, non-standardization of substrate concentration, reaction duration, and temperature and pH conditions, in addition to the fact that different enzyme unit values have been reported by different researchers. Thus, it is difficult to compare the results obtained in the studies and define catalase activity as a diagnostic criterion (Góth et al. 1984). However, some studies were developed to determine and review reference intervals for the activity of this enzyme, in which there was a search for association of enzyme activity with other criteria already established to obtain more references about its clinical relevance and to enable its applicability in clinical diagnoses (Góth et al. 1984; Góth 1991; Vitai and Góth 1997). Furthermore, studies carried out with the purpose of analyzing catalase activity often use a control group and a group with a disease or condition of interest (Guo et al. 2018; Zińczuk et al. 2019).

Therefore, considering the relationship of ROS overabundance in numerous diseases, the need to neutralize the harmful effects caused by free radicals, and the

important antioxidant role played by catalase in hydrogen peroxide dismutation (Galasso et al. 2021), the determination of catalase activity is pointed out as a relevant and applicable biomarker in the assessment of the oxidation-reduction state in different types of biological samples from human beings (Grilo et al. 2020).

Glutathione Peroxidase (GPx)

Characterization and Historical Basis

The enzyme glutathione peroxidase (GPx) is known to play a crucial role in inhibiting lipid peroxidation, and therefore protecting cells from damage caused by oxidative stress. GPx is a family consisting of at least eight isoenzymes known in humans (GPx1–8). Of these, five are selenium dependent, that is, selenoproteins that catalyze the reduction of hydrogen peroxide, lipid hydroperoxides, and hydroperoxides by reducing glutathione (GSH) (Flohé 1999) (Fig. 3).

GPx-1, the first glutathione peroxidase identified in mammals, was first characterized in 1957 as an erythrocyte enzyme that protects hemoglobin from oxidative damage (Mills 1957). It is an enzyme found in all types of cells and is also known as cytosolic or cellular GPx; its function is to catalyze or reduce a large amount of free organic hydrogen peroxides and hydroperoxides, transforming them, respectively, into water and alcohol, by reducing glutathione (GSH). However, GPx-1 cannot metabolize fatty acid hydroperoxides to phospholipids unless accompanied by phospholipase activity to release the fatty acids (Grossmann and Wendel 1983).

A second form of GPx that occurs in the cytosol was called gastrointestinal GPx, now known as GPx-2, an enzyme very similar to GPx-1 in amino acid and nucleotide sequence, both of which have similar substrate specificity for hydrogen peroxide reduction or hydroperoxides. In rats, it is predominantly found in the

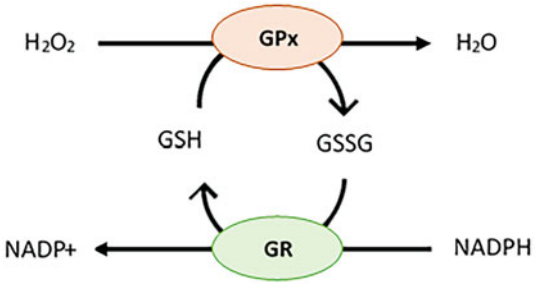

Fig. 3 Schematic model of redox pathways present in the maintenance of cofactors necessary for the activity of glutathione peroxidase
Legend: Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide and lipid hydroperoxides at the expense of reduced glutathione (GSH), which is oxidized to form oxidized glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG into GSH using NADPH as a source of reducing equivalents.

gastrointestinal system and in humans in the intestine and liver (Chu, Doroshow and Esworthy 1993). It protects against hydroperoxides passing through the gastrointestinal tract and reacts with peroxides resulting from by-products of digestive metabolism of food and xenobiotics in the liver. These actions contribute to a potential antimutagenic effect related to hydroperoxides and may protect the gastrointestinal tract by reducing the risk of developing malignant neoplasms (Flohé 1999). GPx-2 also serves as a body reserve of selenium and can be used for immediate selenium needs (Holben and Smith 1999).

GPx-3 or extracellular glutathione peroxidase, identified in 1986 by Takahashi et al. (1987), appears to be synthesized primarily in renal tubular cells. Its function is to serve as an antioxidant barrier for tubular filtrate and protect epithelial cells from oxidative damage caused by free radicals in the form of peroxides. It is also the only glutathione peroxidase present in breast milk (Flohé 1999).

GPx-4 or GPx phospholipid hydroperoxide, identified by Ursini et al. (1985), was the fourth selenium-dependent glutathione found in mammals. Unlike other GPxs, which have a tetrametric structure, this enzyme is structurally a monomer. Its function is to neutralize the oxidative action of fatty acid hydroperoxides in the cell membrane, which are reduced and esterified to phospholipids. GPX-4 can also use hydrogen peroxide as a substrate, as well as a wide range of other lipid hydroperoxides in addition to phospholipid hydroperoxides. This enzyme also acts to reduce cholesterol hydroperoxides and cholesterol esters in membranes and in low-density lipoproteins (LDL), as well as blocking lipid peroxidation in the metabolism of eicosanoids (Flohé 1999).

Determination Methods

Two types of methods are used to determine glutathione peroxidase activity, one of which involves direct measurement of GSH produced at fixed time periods by polarographic GSH analysis. The other is the dithionitrobenzoic acid method, which is based on the ability of reducing glutathione with NADPH to regenerate GSH from GSSG. The reduction in NADPH is continuously measured by spectrophotometry while the concentration of GSH in the enzyme cycle remains essentially constant (Wendel 1981).

Importance and Applications to Prognosis, Other Diseases, or Conditions

The clinical importance of glutathione peroxidase has been demonstrated by a number of studies. Chabory et al. (2010) postulated that individuals with lower enzyme activity have less protection against oxidative damage to cell membrane fatty acids, functional proteins, and against neurotoxic damage. Forgione et al. (2002) hypothesized that the deficiency of the GPx-1 isoenzyme directly induces an increase in vascular oxidative stress, with consequent endothelial dysfunction.

GPx-1 is a crucial antioxidant in preventing the harmful accumulation of intracellular hydrogen peroxide and has been found to be more effective than catalase in removing intracellular peroxides. Its activity is involved in the prevention of several chronic diseases, including cancer and cardiovascular diseases (Lubos, Loscalzo and Handy 2011).

In carcinogenesis, reduced levels of GPx-1 may contribute to the initiation of cancer development and, in later stages of cancer, enzyme deficiency may increase proliferative responses. In contrast, adequate levels of GPx-1 can help prevent DNA oxidation and reduce the inflammatory process, in addition to blocking cell death by apoptosis, leading to increased survival of transformed cells (Brigelius-Flohe and Kipp 2009).

In a prospective study of patients with coronary heart disease, GPx-1 activity and the risk of future cardiovascular events were inversely related to erythrocyte GPx-1 activity levels (Blankenberg et al. 2003). And, in a follow-up study, individuals with high GPx-1 activity were more protected from the deleterious effects of hyperhomocysteinemia (Schnabel et al. 2005). Further, those with the lowest GPx-1 levels were at increased risk for cardiovascular events (Zuzak et al. 2017).

The GPx-4 enzyme was considered the main inhibitor of the ferroptosis process, a non-apoptotic form of cell death. Ferroptosis is characterized by increased levels of lipid hydroperoxides and iron overload. This pathway was first described in cancer cells and has since been identified in hippocampal and kidney cells. Given the irreplaceable role of GPx-4 in the reduction of lipid hydroperoxides in biological membranes, the inhibition of the ferroptosis process by GPx-4 provides protection mechanisms against physiological and pathological processes related to lipid peroxidation, including aging, neurodegeneration, and atherogenesis (Yang et al. 2014; Conrad et al. 2016).

It is a fact that the GPx family exerts many cellular functions, from protection against oxidative damage to the control of cellular processes, such as apoptosis, growth and signaling, modulation of intracellular levels of hydrogen peroxide, and the general intracellular redox balance, which makes this enzyme antioxidant as a crucial element in the process of preventing chronic and degenerative diseases.

Superoxide Dismutase (SOD) Enzyme

Characterization and Historical Basis

Superoxide dismutase is one of the most effective intracellular antioxidant enzymes. It provides the first line of defense against the toxic effects of the accumulation of reactive oxygen species, being responsible for the detoxification of the organism (Gill and Tuteja 2010). SOD proteins are metalloenzymes that preserve up to 97% the targets of superoxide anion attack. They are part of the primary and fundamental line of the enzyme defense system, and are present in practically every cell in the body (Perry et al. 2010).

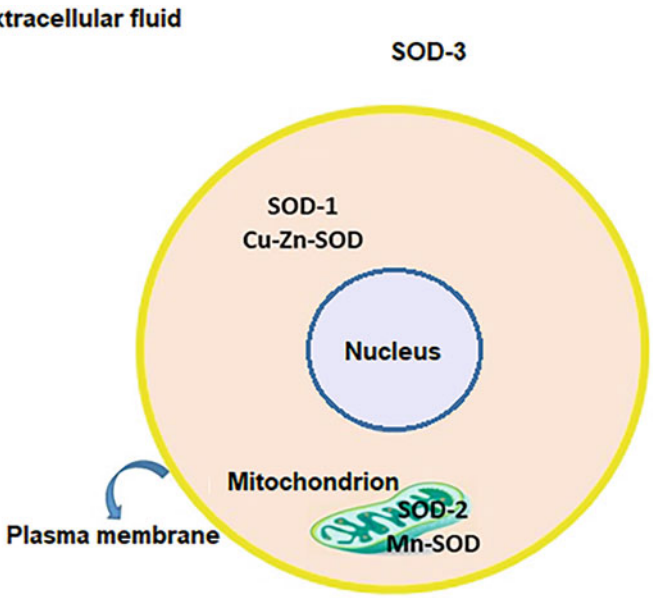

Fig. 4 Cellular distribution of SOD isoforms
Legend: This figure shows the cellular location of the three isoforms of the superoxide dismutase enzyme found in eukaryotic organisms. The SOD1 enzyme found in the cytoplasm; the SOD2 enzyme present in mitochondria; and the SOD3 enzyme found in the extracellular environment. Source: Prepared by the authors (2021).

Historically, superoxide dismutase was first discovered by McCord and Fridovich (1969) as an erythrocyte protein capable of catalytically scavenging superoxide radicals. This metalloenzyme can be found in all cellular compartments capable of producing ROS, being classified into three isoforms (Fig. 4), according to its metallic cofactor: 1. the Cu-Zn-SOD (SOD1) form dependent on copper and cinc, which is located in the cytoplasm, and whose activity is not affected by oxidative stress; 2. the Mn-SOD (SOD2) form, dependent on manganese, which can be found in mitochondria and whose activity increases with the presence of oxidative stress; and 3. the form called SOD3, which can be located in the extracellular environment. There is also Fe-SOD (iron-dependent) form, found in some bacteria and in plant chloroplasts. SOD isoforms act by a unique mechanism of dismutation of the superoxide anion, producing hydrogen peroxide, which is less potent compared to the superoxide anion (Dalvi et al. 2013).

Superoxide dismutase enzymes convert the superoxide anion (O_2•-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), playing a critical role in inhibiting the process of peroxynitrite formation and endothelial and mitochondrial dysfunctions. These proteins act by reducing and oxidizing the superoxide through a reaction called disproportionation (Sheng et al. 2014):

$$\text{Superoxide disproportionation: } 2O_2\bullet\text{-} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

The superoxide anion (O_2•-) is a type of reactive radical species produced during normal aerobic metabolism. It can be toxic in some systems; however, it is also involved in chemical signals. Since O_2•- can be harmful, the dismutation reaction catalyzed by SOD is critical to life. Superoxide dismutase provides protection against oxidative stress caused by aerobic metabolism within the cell's generator, the mitochondria. Reactive oxygen species such as superoxide can damage cells. Antioxidant catalysts, such as SOD enzymes, help to regulate and "deactivate" ROS, turning them into less harmful molecules such as oxygen (O_2) and hydrogen peroxide (H_2O_2), as illustrated in the reaction above (Sheng et al. 2014).

The methodology of analysis of SOD is based on the principle that the xanthine oxidase enzyme, which has xanthine as its substrate, in the presence of O_2, generates a superoxide anion-producing system. These ROS, in the presence of a chromophore, reduce this compound to form a formazan derivative. However, this reduction is inhibited in the presence of superoxide dismutase (Vasconcelos et al. 2007).

Determination Methods

Most procedures for determining superoxide dismutase activity are performed by indirect assessment methods, usually by addition to the erythrocyte of the xanthine system – xanthine oxidase as a source of superoxide anion (O_2•–) and a compound that is reduced by it, as, for example, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazol chloride (INT, $C_{19}H_{13}ClIN_5O_2$ .x H_2O 505.7 g/mol). The superoxide transfers an electron to the INT and produces formazan, detected in a spectrophotometer at 505 nm. The action of the enzyme is measured by the degree of inhibition of the reaction to which the erythrocyte was submitted and the values are expressed in units per gram of hemoglobin (Hb) (U/g Hb). The inhibition of chromogen formation is proportional to the SOD activity present in the sample. In this technique, a 50% inhibition is considered as a unit of SOD (Misra and Fridovich 1972; Gaeta et al. 2002).

In the methodology established by Oyanagui (1984), the determination of the blood SOD activity is determined through the use of xanthine oxidase, which catalyzes the oxidation reaction of hypoxanthine and xanthine, and as a consequence generates superoxide in the presence of oxygen. In this process, the increase in SOD activity in the samples causes a decrease in the superoxide concentration and a reduction in product color (naphthyl-ethylenediamine and sulphanilic acid).

In the study by Romuk et al. (2019), another method was presented where the expressions of the isoenzymes SOD, MnSOD, and Cu-Zn-SOD were measured using potassium cyanide (KCN) as an inhibitor of the isoenzyme Cu-Zn-SOD. SOD 1 activity was determined by the difference between the total SOD activity and the MnSOD isoform activity. Enzyme action was measured against a blank probe (containing double-distilled water). SOD activity was represented in nitrite units (NU) per milliliter of serum. A NU indicates 50% inhibition of nitrite ion formation under the conditions of the method used.

The superoxide dismutase enzyme plays a fundamental role in the modulation of reactive oxygen species, and its induction is related to the extension of redox imbalance in the cell. An increased SOD activity represents an adaptive response to the production of superoxide anions and instability in the relationship between SOD and GPx activity, which may represent a marker of oxidative stress in cells (Mehta and Li 2001).

Importance and Applications to Prognosis, Other Diseases, or Conditions

SOD is essential in the body's defense against reactive oxygen species, as it acts in the removal of superoxide radicals. However, with the natural aging process, the production of antioxidant enzymes decreases and, consequently, increases the chance of imbalance between the action of antioxidant and pro-oxidant mechanisms, with more intense action of free radicals and greater risk of cell damage (Vasconcelos et al. 2014).

In this sense, the theory of aging suggested by Denham Harman (1956) states that endogenously produced reactive oxidants cause oxidative damage to macromolecules, which cumulatively give rise to the aging phenotype. Over the years this theory has undergone modifications and challenges. However, one fundamental principle has remained unchanged, that is, the balance between antioxidant and oxidant compounds is disrupted with aging and results in the accumulation of oxidative damaged macromolecules in the elderly (Zhang et al. 2015).

New choices and changes in dietary patterns and lifestyle can reduce the risk of developing some diseases and contribute to increased longevity (Kruk 2014). Diet can influence the oxidative status, since many foods have in their composition compounds with antioxidant action such as vitamins A, C, and E, minerals such as selenium, zinc, copper, and manganese, as well as carotenoids and phenols. They are also sources of pro-oxidant substances, such as iron, polyunsaturated fatty acids, and others (Vásquez et al. 2011). Regular consumption of vitamin-rich fruits and vegetables increases antioxidant potential, particularly in the blood (Wahlqvist 2013).

The antioxidant compounds present in food act together and interact with each other, enhancing their functions. Zinc and copper are part of the cytosolic superoxide dismutase enzyme Cu-Zn-SOD, while manganese acts as a metallic cofactor of superoxide dismutase in the mitochondrial matrix to Mn-SOD (Vásquez et al. 2011).

To provide protection against oxidative stress, the ingestion of antioxidants is essential to help repair damage caused by oxidizing agents (Selvaraj et al. 2019). In these reactions, several micronutrients play an important role, including zinc, which, in addition to participating in the structure of the SOD1 isoform, is essential for the normal function of the endogenous antioxidant system, as it acts as a potent stabilizer of cell membranes, structural proteins, and of cell signaling (Micheletti et al. 2001).

A study with male triathletes compared the effects of two dietary interventions on oxidative stress and demonstrated that, after 14 days of regular antioxidant diet and

antioxidant-rich diet, there was an increase in SOD activity, although no changes in antioxidant capacity were observed enzymatic, and GPx activity. Furthermore, as superoxide dismutase activity increased, protein damage decreased (Schneider et al. 2018).

In a study evaluating the expression of the enzymes Mn-SOD and Cu-Zn-SOD in gastric and esophageal carcinomas and their relationship with the clinical course of the disease, Miranda et al. (2000) found that the excess of free radicals and the increase in the expression of SOD2 are associated with carcinogenesis of esophageal, gastric, and colorectal cancers. Oxidative stress allows malignant cells to become more susceptible to SOD2 inhibition than normal cells.

In addition, some studies describe the relationship between the activity of SOD1 (Cu-Zn-SOD) and a zinc- and copper-dependent isoform, with the development of some neurodegenerative diseases, such as amyotrophic lateral sclerosis, which consists of irreversible paralysis of the motor system, muscle atrophy, and respiratory failure (Tsitkanou et al. 2016; Geevasinga et al 2016).

Impaired SOD expression in humans has also been associated with several chronic diseases, including ovarian cancer and diabetes mellitus (Koltuksuz et al. 2000). Furthermore, an increase in SOD activity has also been observed after exercise training as a consequence of the oxidative stress promoted by exercise (Tiidus and Houston 1994). In this context, as a potential antioxidant enzyme in the body present inside and outside cells, SOD has a fundamental action against oxidative stress, whose activity can contribute to the body's protection and to reduce the chance of developing various diseases.

Malondialdehyde (MDA)

Characterization and Historical Basis

Malondialdehyde (MDA) is an aldehyde formed at the end of the oxidation of polyunsaturated fatty acids (Fig. 5), such as arachidonic acid, through enzymatic processes involving the biosynthesis of thromboxane A2 (TXA2) or by non-enzymatic processes through endoperoxides produced during lipid peroxidation (Ayala et al. 2014).

It is a small, reactive molecule composed of three carbon atoms with two aldehyde groups in position 1 and 3. It has tautomeric property, that is, it is pH dependent (Morales and Munné-Bosch 2019), being reactive and volatile in acidic medium, and under heating conditions it easily reacts with a variety of nucleophilic agents producing chromogens with high absorptivity (Lima and Abdalla 2001).

MDA can be enzymatically metabolized by some enzymes, such as cyclooxygenases, or react with DNA. It is considered a good marker of oxidative stress, being able to cause damage and form adducts which can become mutagenic, and with irreparable alterations (Ayala et al. 2014; Gegotek and Skrzydlewska 2019).

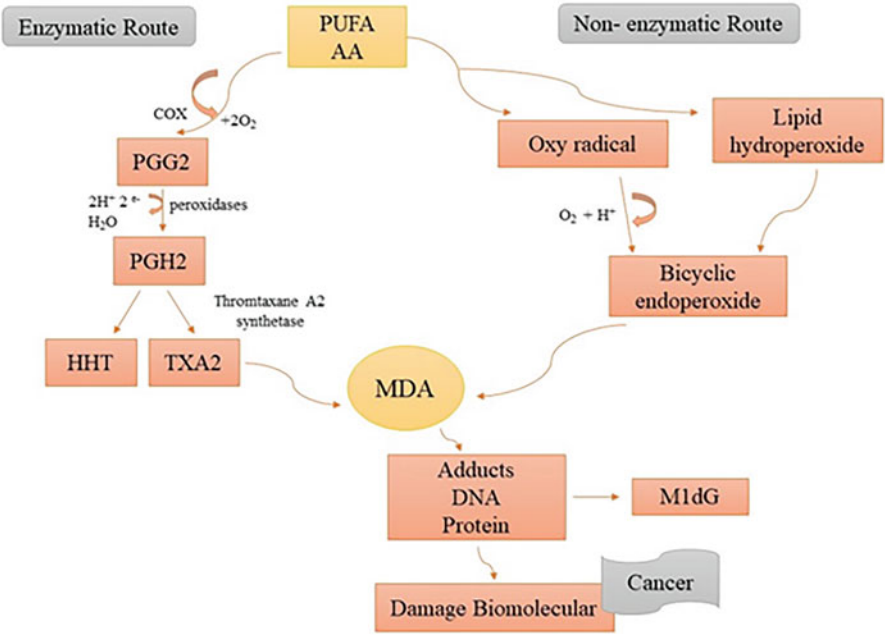

Fig. 5 MDA formation by enzymatic and nonenzymatic pathways
Legend: The formation of MDA occurs by enzymatic pathway from arachidonic acid (AA) with the participation of cyclooxygenases (COX) with the formation of cyclic endoperoxides (PGG2) and thromboxane (TXA2); the nonenzymatic pathway involves the participation of oxygen radicals and lipid hydroperoxides to form bicyclic endoperoxides. MDA can form adducts with DNA or proteins such as M1dG and cause cell damage inducing some diseases such as cancer. Source: Prepared by the authors (2021)

Determination Methods

Several methods are applied for the determination of MDA, including gas chromatography (GC), liquid chromatography with UV or fluorescence detection, and mass spectrometry (Morales and Munné-Bosch 2019).

The most accessible and used technique was introduced by Yagi (1976). It consists in measuring MDA through a reaction with thiobarbituric acid (TBA) at low pH and high temperatures for the production of products with measurement of absorbance by spectrophotometry at 532 nm (Lima and Abdalla 2001; Grotto et al. 2008), known as thiobarbituric acid reactive substances (TBARS) test (Fig. 6). However, this method demonstrates little specificity for the measurement of MDA, as TBA is able to react with other aldehydes, such as protein and carbohydrate degradation products, and which can also show a pink color and be confused (França et al. 2013).

Given this limitation, methods have been developed to increase reproducibility and specificity for detection of MDA (Ghani et al. 2017). One of these methods is based on the reaction of MDA with TBA combined with the HPLC technique in human plasma in vitro, a method that appears to be reliable and reproducible. However, it requires

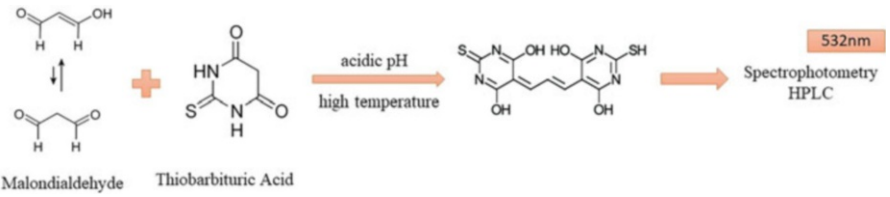

Fig. 6 Thiobarbituric acid reactive substances (TBARS) method for detection of MDA
Legend: Reaction of MDA with thiobarbituric acid at acidic pH at high temperatures with the formation of pink-colored chromogens that can be quantified by spectrophotometry (523 nm) or HPLC. Source: Prepared by the authors (2021)

individual processing of samples, which still seems to be unreliable for in vivo conditions, which makes the technique less performed (Breusing et al. 2010).

In addition, there are commercial kits, whose determinations are easy to reproduce, that use the colorimetric method and the ELISA technique for their quantification, but which lack specificity and standardization (Frijhoff et al. 2015).

There is a difficulty in directly quantifying MDA with these mentioned methods, as MDA has a great capacity to bind to proteins and other biomolecules, not bringing a specific result on the marker, leading underestimation in the quantification of MDA. Despite this, the TBARS method is still widely used due to its low cost and easy reproduction (França et al. 2013).

According to Antunes et al. (2008), there is no standardization for reference values for this marker. In some studies carried out in healthy populations, the values showed great variation. In this sense, Londero and Greco (1996) found mean values of 0.85 ± 0.25 μM in plasma from 104 healthy blood donor patients aged between 18 and 65 years through a technique that consisted of mixing phosphoric acid with TBA and HPLC analysis. In a different way, Pilz, Meineke, and Gleiter (2000) found higher MDA values in human plasma samples from 12 healthy, nonsmoking men aged between 18 and 30 years (2.16 ± 0.29 μM), based on determination in the derivatization with 2,4-dinitrophenylhydrazine (DNPH) in an acidic medium using the HPLC technique. And, Mao et al. (2006), in determinations carried out in plasma of 15 healthy men, aged 20–24 years, found lower values (0.42 ± 0.02 μM) for total MDA, determined through derivatization of MDA with 9-fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) in acidic medium using the HPLC technique.

It is noteworthy that the possibility of comparing values obtained in different studies depend on the technique used to analyze the biomarker and the conditions under which the experiment is carried out, which reinforces the need to standardize values for use in different populations and clinical conditions.

Importance and Applications to Prognosis, Other Diseases, or Conditions

Reactive oxygen species (ROS) are produced in metabolic processes and, when produced in excess, can react with polyunsaturated fatty acids (PUFA) in a lipid peroxidation (LP) process, in which unsaturated fatty acids receive molecular

oxygen producing lipid hydroperoxides as a primary product, and aldehydes (such as MDA) as final products (Grotto et al. 2008; Leon and Borges 2020).

Lipid peroxidation is able to change the permeability of membranes, interfering with the control of the input and output selectivity of nutrients and other substances through the membrane, in addition to producing modifications in the DNA (Lima and Abdalla 2001). Thus, the study of this process is important in the health area and, especially, for the nutrition, pathophysiology, and food sciences.

An applicability of MDA involves food quality, as lipid oxidation causes food deterioration with changes in appearance, odor, aroma, and nutritional loss of fat-soluble vitamins, and, in some cases, formation of toxic compounds (Ghani et al. 2017; Baron et al. 2020).

Increased MDA concentrations in plasma and urine may be associated with carcinogenesis, as MDA is able to react with nucleic acid bases, resulting in the formation of adducts, such as the 3-(2-deoxy-β-d-erythro-pentafuranosyl) pyrimido [1,2-α]purin-10(3H)-one deoxyguanosine (M1dG) adduct (Cai et al. 2012).

M1dG is a mutagenic agent in bacteria and mammalian cells, and it is an adduct found in the genomic DNA of healthy individuals. If repair does not occur, cells, such as those of the intestine, can undergo apoptosis or genetic mutation. In addition, M1dG can be identified, albeit at low concentrations, in the urine of healthy individuals. So, it can be a useful biomarker of DNA damage linked to oxidative stress (Otteneder et al. 2006; Cai et al. 2012).

In a retrospective study, Didziapetriene et al. (2014) linked MDA concentrations with long-term survival in breast cancer patients and found that increased MDA concentrations were associated with lower survival rates in stage I and II patients, but not with more advanced stages of the disease. Leung et al. (2008) found higher concentrations of MDA in patients with advanced and inoperable colon cancer than in patients with primary-stage cancer.

The role of antioxidant foods and nutrients in improving parameters related to oxidative stress has been extensively studied. Akrami et al. (2018) carried out a controlled randomized clinical trial with 60 patients with metabolic syndrome who consumed flaxseed oil and sunflower oil in different groups and observed a decrease in MDA only with flaxseed oil supplementation.

The relationship between MDA concentrations and nutritional status has also been analyzed. A study by Viana et al. (2020) with 51 adult male patients who were candidates for liver transplantation showed a negative correlation between plasma MDA concentration and body mass index (BMI) in patients with liver cirrhosis. Furthermore, it was demonstrated that the consumption of antioxidant nutrients was inadequate, indicating that the nutritional deficit can also contribute to an increase in oxidative stress markers.

Mini-Dictionary of Terms

- **Metalloenzymes**. Metallobiomolecules that contain at least one metal ion in their active site with a structural and functional role in biological systems. They act mainly by catalyzing oxidation-reduction reactions.

- **Dismutation**. Chemical reaction in which one part of the substances is oxidized and the other is reduced.
- **Reactive oxygen species (ROS)**. Substances produced in the respiratory chain which, when in excess of antioxidant systems, can damage biological tissues. The main ROS are hydroxyl radicals, superoxide, singlet oxygen, and hydrogen peroxide.
- **Single-nucleotide polymorphism**. Mutations in a single base in the DNA molecule sequence, that is, mutations in single base in the chain of nitrogenous bases (adenine, cytosine, thymine, and guanine).
- **Xenobiotics**. Chemical compounds foreign to an organism which does not produce or is not expected to produce such compounds. The term is also applied to substances present in an organism in higher than normal concentrations.

Key Facts of Oxidative Stress in Chronic Noncommunicable Diseases

Chronic noncommunicable diseases (CNCDs) including cardiovascular diseases, diabetes mellitus, systemic arterial hypertension, obesity, and cancer are considered the main causes of mortality in the world.

The causes of these diseases are also related to lifestyle such as inadequate diet, smoking, physical inactivity, and alcoholism.

In all these pathologies, oxidative stress is part of the development of the disease and of its causes.

Oxidative stress corresponds to a metabolic imbalance that generates the formation of free radicals whose chronic and progressive action leads to the development of different CNCD.

To treat these chronic diseases and oxidative stress, it is necessary to adopt a healthier lifestyle.

Summary Points

- Superoxide dismutase enzymes are metalloenzymes responsible for dismuting the superoxide radical into oxygen and hydrogen peroxide.
- SODs are part of the primary and fundamental line of the enzymatic defense system and are present essentially in every cell in the body.
- SODs are classified according to the metal incorporated in their structure or where they are found: copper and zinc (Cu-Zn-SOD), manganese, or iron (Mn-SOD/Fe-SOD).
- Catalase, an enzyme that is part of the antioxidant defense to protect cells from the harmful effects of free radicals, works through a reaction that converts hydrogen peroxide into water and molecular oxygen.
- Malondialdehyde (MDA) is a secondary compound of the lipid peroxidation process.

- The method based on the determination of substances reactive to thiobarbituric acid is the most used for quantification of MDA.
- GPx is a family consisting of at least eight isoenzymes known in humans (GPx1–8). Of these, five are selenium dependent, that is, selenoproteins that protect against oxidative damage.
- GPx acts to reduce hydrogen peroxide, lipid hydroperoxides, and hydroperoxides by reducing glutathione (GSH).
- GPx-1, the first glutathione peroxidase identified in mammals, is found in all cell types and its action prevents the accumulation of intracellular hydrogen peroxide.

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The Oxidative Balance Score as a New Nutritional Scoring System

41

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Abstract

Oxidative stress can provide a ground for the development of cardiometabolic disorders. In such conditions, increasing the power of the antioxidant agents to rebalance pro- and antioxidant markers is necessary to improve the status and prevent the progress of complications. Dietary patterns and lifestyle can play the key roles in maintaining the balance between pro- and antioxidant markers.

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Consumption of nutrients and food full of the anti-oxidative components is likely to be one of the main defensive methods. Although biochemical tests can show pro-oxidant and antioxidant concentrations, they are expensive. Therefore, using a valid, accessible, and inexpensive alternative tool containing main parameters affecting oxidative status will be helpful to clarify the status of body in this regard. One of the new dietary indices is the "Oxidative Balance Score" that will be introduced in the present chapter.

Keywords

Oxidative stress · Cardiometabolic risk factors · Diet · Lifestyle · Oxidative Balance Score

Abbreviations

CRP	C-reactive protein
CVDs	Cardiovascular diseases
DASH	Dietary Approaches to Stop Hypertension
ER	Endoplasmic reticulum
FIP	F2-isoprostanes
FOP	Fluorescent oxidation products
GGT	γ-Glutamyltransferase
GP	Glutathione peroxidase
MDA	Malondialdehyde
NSAIDs	Non-steroidal anti-inflammatories
OA	Osteoarthritis
OBS	Oxidative Balance Score
PUFAs	Polyunsaturated fatty acids
RNS	Nitrogen species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SFAs	Saturated fatty acids
SOD	Superoxide dismutase
SREBP	Sterol regulatory element binding protein
TFAs	Trans fatty acids
WHO	World Health Organization

Introduction

Cardiovascular diseases (CVDs), including coronary artery diseases, vascular disease, heart attack, stroke, and other cardiac and vascular conditions, are the major causes of mortality globally (Mensah et al. 2019). The incidence of CVDs is remarkably increasing, particularly in young individuals that may be due to unhealthy shift in lifestyle (Sharifi-Rad et al. 2020). Cardiometabolic risk factors, such as obesity, dyslipidemia, inflammation, hyperglycemia, and atherosclerosis,

can emerge following adherence to unhealthy dietary patterns and low physical activity (Badimon et al. 2019). As the two above mentioned parameters are modifiable risk factors for preventing the occurrence of CVDs, the identification the effective strategies in this regard can be helpful to prevent and manage cardiometabolic disorders and related physical and psychological complications (Sharifi-Rad et al. 2020).

Evidence showed the beneficial effects of healthy dietary patterns, such as Dietary Approaches to Stop Hypertension (DASH) (Bricarello et al. 2018; Tiong et al. 2018; Fagherazzi et al. 2020), Mediterranean diet (D'Innocenzo et al. 2019; Martín-Peláez et al. 2020; Delarue 2021), and plant-based diets (Singh et al. 2019; Dąbrowska et al. 2020) on cardiometabolic parameters. Studies have shown that oxidative stress is suspected to be involved in the pathogenesis of cardiometabolic disorders (Vassalle et al. 2020; Jakubiak et al. 2021). Thus, diet rich in foodstuffs with antioxidant characteristics seems to play the key roles in preventing the progress of metabolic disorders (Narmaki et al. 2015; Tiong et al. 2018; Singh et al. 2019).

An imbalance between anti-oxidative and pro-oxidative factors in favor of pro-oxidants can lead to oxidative stress (Jakubiak et al. 2021). In fact, oxidative stress is a multifactorial process that may occur due to several factors, such as smoking, unhealthy diets, severe exercise, and taking of some medicines. Reactive oxygen species (ROS), nonradical molecules, and highly reactive radical agents produced in the course of oxygen metabolism, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are placed in pro-oxidative category. On the other hand, glutathione peroxidase (GP), superoxide dismutase (SOD), and catalase are key antioxidant enzymes to tackle the oxidative status. Apart from enzymes, some components with nonenzymatic properties, including ascorbic acid, beta-carotene, tocopherols, tocotrienols, and glutathione, have shown anti-oxidative actions (Jakubiak et al. 2021). The imbalance can damage macromolecules and cells. Consequently, a ground for development of cardiometabolic disorders is provided (Vassalle et al. 2020). In such conditions, increasing the power of antioxidant agents to rebalance the pro- and antioxidant markers is necessary to improve the status and prevent the progress of complications. As explained earlier, consumption of nutrients and food full of anti-oxidative components is likely to be one of the main defensive methods for the said conditions (Carocho et al. 2018).

For instance, among dietary factors, nutrients like flavonoids, carotenoids, zinc, selenium, tocopherols, and vitamin C can act as antioxidants, while saturated fatty acids, polyunsaturated fatty acids, and iron can be recognized as pro-oxidant factors. In addition, fruits and vegetables are rich sources of antioxidant nutrients, while red meat and alcohol are deemed as pro-oxidants (Hernández-Ruiz et al. 2019). Paying attention to a whole diet, however, is more practical and effective than focusing on one or more certain food items due to the interactions among nutrients. To examine the properties of dietary patterns, a number of dietary indices (Kong et al. 2015; Namazi et al. 2017, 2018; Zamani et al. 2021) are developed the attribute scores to each type of diet and make it possible to compare the effects of the different dietary patterns considering various foodstuffs and nutrients on health. One of new dietary indices is the "Oxidative Balance Score" aimed to be introduced in the present chapter.

Oxidative Balance Score

Oxidative Balance Score (OBS) is a new index considering various antioxidants and pro-oxidative parameters. Obviously, in this score, the antioxidant agents can positively affect the oxidative status, whereas oxidative factors can negatively affect the oxidative status. As a consequence, lower OBS reflects a predominance of pro-oxidants to anti-oxidative agents (Hernández-Ruiz et al. 2019). It was indicated that a higher OBS representing a predominance of antioxidant vs. pro-oxidant agents was strongly and inversely associated with the serum levels of γ-glutamyltransferase (GGT). Notably, GGT is a biomarker of oxidative stress (Cho et al. 2018). Thus, it seems that the score can be used instead of the oxidative stress markers to explore the association with some diseases.

In the first OBS, a combination of two dietary antioxidants (vitamin C and beta-carotene) and only one dietary pro-oxidant (iron) was considered (Van Hoydonck et al. 2002). Although this score was adversely associated with a lower risk of mortality in smokers, it was not being able to sufficiently reflect the overall exposure to pro- and anti-oxidants. Accordingly, several OBSs have been developed using multiple approaches, containing the different factors, such as dietary factors, medication, lifestyle parameters, biomarker components, and genetics. Moreover, there are differences not only in the factors considered in this score, but also the modeling and defining of the score are varied (Hernández-Ruiz et al. 2019). A review study performed by Hernández-Ruiz et al. showed that 21 OBSs have been introduced so far (Table 1). The components considered in the score ranged from 3 to 20 items. Since OBSs are a combination of antioxidant and pro-oxidant components, all the constructed OBSs included both of them, although there are considerable variations among the scores. In addition, the total score varied between 0 and 60 (Hernández-Ruiz et al. 2019).

Among dietary antioxidants, approximately all included vitamin E, vitamin C, and beta-carotene, and a considerable proportion of OBSs included selenium, lutein/zeaxanthin, and lycopene, whereas fewer OBSs considered vitamin B9, vitamin D, retinol, calcium, zinc, and total catechin. Only one OBS included heme iron. Regarding biomarker components, four OBSs assessed the antioxidant and pro-oxidant status with these factors and a single OBS accounted for antioxidant biomarkers [10]. The scores suggested by Lakkur et al. (2014a) and Goodman et al. (Goodman et al. 2010) considered the greatest number of biomarker components.

In terms of lifestyle components, smoking, physical activity, and obesity were included in some types of scores. Among these three factors, obesity was the most frequent score among the scores ($n = 7$ studies). Moreover, aspirin and NSAID representing medication were observed in 11 OBSs and the genetic variants were involved in only 2 types of scores.

Recent evidence on both pro-oxidants and anti-oxidants involved in the OBS and their effects on health and metabolic status is provided below.

Total fat and fatty acids: Dietary fat play key roles in various pathways and functions in the human body. For instance, it is used as (i) a main metabolic fuel to provide the required calorie, (ii) a necessary precursor of some hormones, and

Table 1 Components and the score range for the OBSs

Number of components	Number of included antioxidants	Number of included pro-oxidants	Dietary components	Biomarkers	Medication	Lifestyle	Score range	Reference
7	4	3	*			*	0–14	Lee and Park (2017)
8	3	5	*			*	0–24	Cho et al. (2018)
15	9	6	*			*	0–30	Wang et al. (2017)
13	10	3	*		*		0–26	Ilori et al. (2015)
14	10	4	*		*	*	0–28	Lakkur et al. (2015)
13	9	4	*	*	*	*	0–26	Annor et al. (2015)
14	10	4	*		*	*	0–28	Kong et al. (2015)
16	10	6	*			*	−6–10	Dash et al. (2015)
13	10	3	*	*	*	*	0–26	Lakkur et al. (2014)
20	14	6	*		*	*	0–60	Lakkur et al. (2014)
6	5	1	*				0–12	Slattery et al. (2014)
14	10	4	*	*	*	*	0–28	Kong et al. (2014)
11	7	4	*		*	*	0–22	Labadie et al. (2013)
15	9	6	*			*	−6–9	Dash et al. (2013)
8	5	3	*			*	0–24	Geybels et al. (2012)
13	10	3	*		*	*	0–26	Slattery et al. (2012)
13	8	5	*			*	0–52	Agalliu et al. (2011)
14	11	3	*	*	*	*	0–28	Goodman et al. (2010)
12	8	5	*		*	*	0–24	Goodman et al. (2008)
12	9	3	*	*	*	*	0–12	Goodman et al. (2007)
3	2	1	*				3–9	Van Hoydonck et al. (2002)

(iii) structural part of the cell wall. However, high intake of total fat and saturated fatty acid (SFA) diets is associated with the risk of NCDs. They can result in unfavorable effects on intestinal microbiota and disturb the metabolic status. Evidence also showed that the replacement of SFA and trans fatty acids (TFAs) with polyunsaturated fatty acids (PUFAs) can improve health and prevent CVDs and other NCDs. Hydrogenated oil, ghee, and animal products including high-fat dairy products and meat are the sources of SFAs and TFAs, while vegetable oils, such as canola, sunflower, and sesame oils are examples of unsaturated fatty acids (Wolters et al. 2019). Evidence suggests that the type of consumed fat is more important than how much total fat is consumed in terms of keeping health or preventing complications of diseases. The elimination of industrially produced trans fatty acids is strongly recommended, particularly for prevention of CVDs and their related complications. This type of fat can also increase insulin resistance and provide a ground for diabetes mellitus (Forouhi et al. 2018). Interestingly, industrial TFA, not ruminant *trans* fatty acid (found in natural animal products such as dairy products and red meat), can increase inflammatory parameters and endoplasmic reticulum (ER) stress. However, *cis*-unsaturated fatty acids play protective roles against inflammation and ER stress. Furthermore, industrial TFAs greater than *cis*-unsaturated fatty acids and SFAs increase fat storage in the liver and adipose tissue. Such fatty acids also stimulate the cholesterol synthesis pathway through increasing the activity of sterol regulatory element binding protein (SREBP) 2-mediated gene regulation and involve in cholesterol synthesis (Oteng and Kersten 2020).

According to the World Health Organization (WHO), the recommended percentages for different types of fatty acids are as follows: SFA $< 10\%$ and TFA $< 1\%$ and replacement of both with PUFA when SFA consumption is higher than 10% of total energy intake. Common percentage of dietary fat is 30–35% and it is recommended that it should be less than 35% of total energy intake (Schwingshackl et al. 2021).

Alcohol: Alcohol is a kind of beverage that can induce oxidative stress due to the metabolism of ethanol that involves both microsomal and mitochondrial systems. Its metabolism results in the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). It also increases malondialdehyde (MDA) and decreases GSH concentrations. Following these, biological structures are modified and consequently malfunction of cells and tissues occurred (Das and Vasudevan 2007). Metabolism of ethanol is associated with the activation of NF-κB, production of free radical agents, the stimulation of Kupffer cells, increasing levels of TNF-α, and oxidative stress (Das and Vasudevan 2007).

Ferritin: Iron is an essential mineral for normal growth and proliferation of the cells. However, excess iron can damage cells and provide a condition for metabolic disorders through oxidative stress mechanism (Orino et al. 2001). Ferritin can form an empty shell that is likely to bind equal or less than 4500 atoms of iron. Ferritin is a combination of light and heavy chains and chain ferritin types. Heavy-type ferritin ferroxidase can oxidize iron to Fe3+ following Fe2+ incorporation into ferritin in a short time, while light type ferritin might be responsible for electron transfer. Free iron is toxic and damages the cells. Thus, the role of ferritin is to store excess iron to protect the cells (Orino et al. 2001). Apart from taking high dosage of iron

supplements for a long time, rheumatologic diseases, malignancy, and infection are other reasons for increasing iron storage in the body that may be reflected by high levels of ferritin in biochemical tests. It disturbs the balance between pro-oxidant and anti-oxidant agents and damages the cells (Moore Jr et al. 2013). In these conditions, CVDs, some types of cancers, dyslipidemia, and other metabolic disorders can occur (Williams et al. 2002).

Smoking: Smoking is one of main risk factors for CVDs and is associated with oxidative stress and affects mitochondrial function and metabolic state. It can also contribute to endothelial dysfunction and hypertension. It can reduce the levels of mitochondrial deacetylase sirtuin-3 and result in hyperacetylation of SOD2 which is a main mitochondrial antioxidant. Therefore, mitochondrial oxidative stress is promoted (Dikalov et al. 2019). It was also reported that the total antioxidant status and vitamins with anti-oxidative properties (vitamin E, vitamin C) are greater in non-smokers than in smokers. Due to this, various diseases can develop (Karademirci et al. 2018).

Obesity: Imbalance between energy intake and energy expenditure results in fat storage and obesity. It is associated with a number of metabolic disorders, such as cardiovascular diseases, atherosclerosis, dyslipidemia, diabetes mellitus, some types of cancer, asthma, and infertility (Church and Martin 2018). Overweight and obesity are the multifactorial or complex metabolic disorders. Evidence accumulation revealed that oxidative stress plays a pivotal role in the development of diseases following weight gain. Obesity can exert systemic oxidative stress via different biochemical mechanisms. Some potential mechanisms are as follows: oxidative phosphorylation, protein kinase C activation, polyol and hexosamine pathways, and superoxide induced following NADPH oxidases (Manna and Jain 2015). In addition, fat accumulation in obesity increases inflammatory parameters, and hormone with inflammatory properties like leptin is increased, and leptin, an anti-inflammatory hormone, is decreased (Karczewski et al. 2018). Accordingly, using therapeutic strategies, such as adherence to low-calorie diets, changes in dietary habits, increased physical activity, and taking anti-obesity medicines, can be helpful for weight loss and prevention of chronic diseases (Ryan and Kahan 2018).

The Effects of Antioxidants Involved in the OBS on Development of the Non-communicable Diseases

Vitamins: Vitamins are a main part of biological system due to their pivotal roles in various biochemical processes and pathways. Vitamins A, C, and E are examples of vitamins with anti-oxidative properties that can detoxify the free radicals. Their deficiencies or even any alterations in their concentrations may disturb metabolic processes (Asmat et al. 2016). Accordingly, healthy diets rich in fruits and vegetables, fish, and olive oil can provide essential amounts of such nutrients. However, in some cases due to taking medication or disease background diet alone is not sufficient and supplements should be added to be sure about the adequate levels of

vitamins in the body. Studies have shown that there is an association between low levels of vitamin C (Wilson et al. 2017), vitamin E (Mullan et al. 2017; Tsou and Wu 2019), and vitamin A (Kim et al. 2017) or its metabolites with chronic diseases and related complications. Additionally, it can be concluded that taking multi-vitamins and minerals can improve glycemic status, lipid profile, and atherogenic indices compared to control group (Jin et al. 2020). Siavash et al. also reported that supplementation with vitamin C similar to gemfibrozil can increase HDL-C concentrations (Siavash and Amini 2014). However, there are controversies in terms of recommendation of taking multi-vitamins and minerals to prevent CVDs. Ingles et al., for instance, reported that although there is a high intake of dietary supplements by the general public, evidence confirmed that the routine supplementation of such supplements is for either CVD prevention or treatment (Ingles et al. 2020). Nevertheless, the intake of vitamins particularly anti-oxidative ones from diet is recommended.

Minerals: The two minerals including zinc and selenium with anti-oxidative characteristics are involved in the OBS. A considerable number of studies have confirmed the anti-oxidative actions of these two nutrients. Zinc is an essential element to maintain the normal structure and physiology of cells. Research showed the protective effects of zinc in coronary artery diseases. Intracellular zinc is involved in redox signaling pathway and ischemia and infarction induce the release of zinc from proteins. Following this, myocardial damage may occur. In these conditions, taking zinc can promote cardiac function and prevent more damages (Little et al. 2010). Selenium is another anti-oxidative element involved in metabolism, such as thyroid hormones, immune system, functions of some organs, and anti-oxidative defense systems (Hasani et al. 2019; Djalalinia et al. 2021).

The links between selenium intake and/or status and CVDs, diabetes mellitus, male fertility, gastrointestinal diseases, and prostate cancer are reported (Fairweather-Tait et al. 2011).

Flavonoids: Flavonoids play a key role in the protection against oxidative stress and they are widely found in fruits, vegetables, cocoa, and tea. They are involved in various biological activities, but their anti-oxidative effects are mainly highlighted in studies. They can strengthen endogenous antioxidant defenses and restore the optimal balance through neutralizing ROS. Metal ion chelating, free radical scavenging, being a substrate for radicals (e.g., superoxide, hydroxyl), a singlet oxygen quenching, and hydrogen donation are actions by which flavonoid show anti-oxidative properties (Adwas et al. 2019).

Physical activity: A number of studies have shown the effects of physical activity on redox homeostasis. Evidence confirmed that inflammation is widely involved in some chronic diseases and is closely associated with oxidative stress. Regular and moderate physical activity can affect oxidative stress through increasing cellular antioxidant defense, while professional severe exercise can change cellular redox homeostasis toward oxidative stress (Kruk et al. 2019).

In Table 2, the oxidative and anti-oxidative components involved in the OBSs are presented.

Table 2 Dietary, biomarkers, lifestyle factors, and medication components in the OBSs (Hernández-Ruiz et al. 2019)

Dietary factors	Biomarkers	Lifestyle factors	Medication
Pro-oxidants	***Pro-oxidants***	***Pro-oxidants***	
Total fat Polyunsaturated fatty acids Saturated fatty acids Iron Red meat	Ferritin	Alcohol Smoking Obesity (high BMI)	
Anti-oxidants	***Anti-oxidants***	***Anti-oxidants***	***Anti-oxidant***
Vitamin C Vitamin E Total carotenoids Lutein α,β-Carotene Lycopene β-Cryptoxanthin Zeaxanthin Flavonoids Glucosinolates Selenium Zinc	α,β-Carotene Lycopene β-Cryptoxanthin Zeaxanthin α-Tocopherol Gamma-tocopherol	Physical activity	Aspirin Non-steroidal anti-inflammatories (NSAIDs)

Oxidative Balance Score, Cardiovascular Diseases, and Their Related Risk Factors

Kim et al. examined the association between the OBS and the risk of cardiovascular disease in subjects with metabolic syndrome. No significant differences were observed between two categories classified by the presence or absence of atheroma. In addition, components of this score showed no association with the presence of atheroma (Kim et al. 2013). Golmohammadi et al. also reported that in subjects with higher OBS, glycemic control is better than other subjects with type 2 diabetes (Golmohammadi et al. 2019). Regarding hypertension, Annor et al. revealed that higher OBS was associated with lower risk of hypertension (Annor et al. 2015). However, no link was found between this score and metabolic syndrome (Noruzi et al. 2021).

Oxidative Balance Score and Cancers

J.Kong et al. reported that the OBS was inversely correlated with colorectal adenoma. The risk of this type of cancer in subjects with higher OBS was about 60% less than those with the lowest ones. In addition, there was an inverse correlation between the score and the biomarkers of oxidative stress (F2-isoprostanes (FIP) and C-reactive protein (CRP)) levels. However, they

found a direct relationship between the OBS and fluorescent oxidation products (FOP) that could not justify this link (Kong et al. 2014). Kong et al. also indicated that higher OBS can decrease all-cause mortality rate and cancer mortality in the US population (Kong et al. 2015). Mao et al. also demonstrated that a predominance of antioxidant over pro-oxidant dietary and lifestyle exposures was inversely correlated with colorectal cancer and related mortality in older women (Mao et al. 2021). Surprisingly, Agalliu et al. revealed that no associations were found between the OBS and the risks for prostate cancer or advanced disease. Among the components of the score, only red meat increased the risk by 44%, while lycopene decreased the risk of prostate cancer by 30% (Agalliu et al. 2011). Similar to this, Lakkur et al. found no significant association between higher levels of the OBS and the risk of prostate cancer (Lakkur et al. 2014a).

Oxidative Balance Score and Mortality

The study by Van Hoydonck et al. showed that mortality rates, especially cancer deaths in smokers who consumed lower intakes of vitamin C and beta-carotene as well as high levels of iron, were greater than others. Recommending greater servings of fresh fruits and vegetables and less meat can be helpful for such individuals to reduce the risk of mortality (Van Hoydonck et al. 2002). Similar to these findings, Fernandez-Lazaro et al. proposed a strong inverse link between the OBS and lower risk of all-cause, CVD, and cancer mortality (Fernandez-Lazaro et al. 2021).

Oxidative Balance Score, Inflammatory Biomarkers, and Related Diseases

The study conducted by Lakkur et al. revealed an inverse correlation between OBS and elevated CRP concentrations. They concluded that this dietary index is likely to reflect inflammation (Lakkur et al. 2012). Mark et al. also reported that a higher OBS is inversely associated with serum levels of interlukin-6 (IL-6) and CRP. However, there was no significant association between the score and IL-6 in subjects with chronic kidney disease (Marks et al. 2018). Regarding rheumatoid arthritis, Choi et al. showed that high OBS was correlated with lower duration of morning stiffness. Furthermore, when the OBS increased, pain and physical function improved in such patients (Choi et al. 2019). However, in patients with osteoarthritis (OA), in the fully adjusted model, the score was not associated with OA. However, it was associated with better quality of life. What it means is that anti-oxidants can improve the quality of life in subjects with OA (Lee et al. 2019).

In addition, an inverse association was found between the score and chronic kidney disease. In other words, the OBS is likely to be independently linked with lower prevalence of chronic kidney disease and recommending food with oxidative properties can prevent this disease (Ilori et al. 2015).

Conclusion

The OBS is a valuable approach to consider various parameters including diet, lifestyle factors, medication, and genetics to realize links and potential synergies and antagonism effects among parameters. It can be an appropriate, simple, and inexpensive tool to examine the oxidative balance in individuals or populations. Due to differences in the mentioned parameters in different societies with various disease backgrounds, using this can be helpful to provide nutritional and medical recommendations to promote health and prevent CVDs and related risk factors and complications.

Applications to Other Diseases or Conditions

In this study, we review evidence on oxidative balance score (OBS) and its association with various chronic diseases and biochemical parameters including oxidative stress and inflammatory parameters. It suggests there is an inverse association between the OBS and the risk of NCDs and mortality. However, no link was found between this index, prostate cancer, and metabolic syndrome. It also showed an acceptable link with oxidative and inflammatory parameters. Therefore, this index can be a useful tool to predict some metabolic disorders such as CVDs, type 2 diabetes, and dyslipidemia in adults. However, its application for children and adolescents has remained unclear.

Mini-dictionary of Terms

NCDs: They are also known as chronic diseases and can occur due to a combination of factors such as genetic, behavioral, physiological, and environmental parameters.

NSAIDs: They are member of a medication category to decrease pain and can prevent blood clots. In higher dosage, they can reduce inflammatory parameters.

Reactive nitrogen species: They are a family of antimicrobial molecules produced from superoxide and nitric oxide.

Reactive oxygen species: They are high reactive agents produced from O_2 which are involved in cell signaling and metabolic pathways.

Sterol regulatory element binding protein: They are transcription factors which are indirectly needed for cholesterol biosynthesis and uptake of fatty acids and their biosynthesis.

Key Facts of Oxidative Balance Score

- The OBS is a combination index to cover the main parameters involved in oxidative stress.
- There is an inverse association between oxidative stress parameters and the OBS.

- There is no association between the OBS and metabolic syndrome.
- There is an inverse association between the OBS, hypertension, and hyperglycemia.
- There is no link between the OBS and prostate cancer.

Summary Point

- The OBS is an appropriate, simple, and inexpensive tool to examine the oxidative balance in individuals or populations.
- The OBS can reflect inflammatory and oxidative stress parameters.
- The OBS can predict a number of chronic diseases.
- The OBS considers a combination of biochemical, genetic, and environmental factors.
- There is limited evidence on the association between the OBS and chronic diseases.

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Urinary Gluten Immunogenic Peptides as a Biomarker in Celiac Patients

42

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Abstract

Celiac disease (CD) is treated with a lifelong gluten-free diet (GFD). Current methods for monitoring GFD conformance, such as dietary questionnaires, serology tests, or clinical symptoms, may be inaccurate in detecting dietary transgressions. Duodenal biopsies are invasive, expensive, and not a routine monitoring technique. This chapter discusses a promising advancement in the development of tests that measure immunogenic gluten peptides (GIP) in stools

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and urine as evidence of recent gluten exposure. GIP determination in urine or stool is a sensitive, reliable, and clinically proven method for the follow-up of patients with CD that ensures adherence to the GFD and is therefore a biomarker of the state of the duodenal mucosa. The introduction of GIP testing as an assessment technique for GFD adherence may help in targeting the most suitable interventions during follow-up and in differentiating symptoms related to CD and other conditions.

Keywords

Celiac disease · Gluten-free diet · Gluten immunogenic peptides · Lateral flow immunoassay · Urine

Abbreviations

Anti-DGP	Anti-deamidated gliadin peptide
Anti-tTG	Anti-tissue transglutaminase
CD	Celiac disease
ELISA	Enzyme-linked immunosorbent assay
EMA	Anti-endomysial antibody
GFD	Gluten-free diet
GIP	Gluten immunogenic peptides
LFIA	Lateral flow immunoassay

Introduction

Celiac disease (CD) is an immune-mediated systemic disorder that affects approximately 1% of most populations. It is characterized by responsiveness to gluten ingestion in genetically susceptible individuals and by the presence of various combinations of clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy (Lebwohl and Rubio-Tapia 2021; Lindfors et al. 2019; Murray et al. 2018; Rej et al. 2020).

CD is managed mainly with a strict lifelong adherence to a gluten-free diet (GFD) (Bernardo and Peña 2012; Caio et al. 2019; Hall et al. 2013). However, full adherence to GFD is very difficult to achieve given the ubiquity of gluten as a common food additive and due to dietary habits, high GFD costs, and social restrictions it imposes on patients.

The goal of GFD is to relieve symptoms, achieve mucosal healing, and prevent complications associated with untreated CD (Ludvigsson et al. 2014). Lack of adherence to a GFD may lead to the deterioration of the quality of life. Long-term side effects of non-adherence to a GFD may be related to a lack of vitamins and fiber in the diet and may lead to symptoms and complications of CD such as worsening of malabsorption, anemia, and osteoporosis; it may even present an increased risk of adenocarcinoma of the small intestine, esophageal cancer, and T cell intestinal lymphoma (Lebwohl et al. 2014).

At least one-third of patients with CD fail to fully adhere to a GFD. In addition, 36–55% of patients who declare full adhesion to a GFD do not achieve histological remission, probably due to inadvertent lapses in daily gluten intake (Barratt et al. 2011; Comino et al. 2016). Thus, it is necessary to develop a reliable tool to confirm inadvertent gluten ingestion in patients with CD under GFD.

Current Methods for Assessing Gluten-Free Diet Adherence

The methods for evaluating GFD adherence include dietary interviews, clinical responses, or CD serological screening tests to confirm decreased levels of anti-tissue transglutaminase (anti-tTG) or anti-deamidated gliadin peptide (anti-DGP) antibodies. However, none of these methods offer either direct or accurate measures of dietary adherence.

Regarding the role of the dietitian, they must recheck the labels of everyday foods and all prescription medications, evaluate frequency and strategies used when dining away from home, and look for sources of cross-contamination at home, including using a separate toaster, clean kitchen counters, and separate cooking and serving utensils and avoiding double dipping in common condiment jars. However, all of this is based on diet questionnaires, which should be standardized to different dietary habits in every country and require an individual structure in every location, although these conditions are never met. Therefore, even a thorough review of eating habits may fail to reveal the ingestion of significant amounts of gluten, which can be unintended or embarrassing for patients with CD when facing the physician or dietician (Leonard et al. 2017).

Clinical response alone is a poor predictor of strictness of adhesion to a diet. CD diagnosis in adults rarely presents with malabsorption syndrome symptoms, and many patients experience only vague or low-level symptoms that they may not recognize until they start a GFD. In addition, patients may be asymptomatic for many years or decades before presenting with a complication of CD, and many patients are asymptomatic or minimally symptomatic at presentation and clearly cannot be followed up using symptom relief as the main determinant of clinical response (Sharkey et al. 2013). Furthermore, the symptoms correlated poorly with villous atrophy. In a controlled study of gluten re-challenge, symptoms were absent in 22% of cases where significant villous atrophy occurred (Lahdeao et al. 2011). Moreover, many of the presenting symptoms of CD overlap with other common conditions such as irritable bowel syndrome, which may also be present and not respond to gluten withdrawal. Therefore, follow-up based on symptoms alone is inadequate.

Regarding serology, several reports have shown time-dependent normalization of anti-tTG and anti-DGP concentrations after instituting the GFD without any necessary correlation with mucosal healing. In the series of the Department of Gastroenterology of the University of Cambridge, only 43.6% of patients with persistent villous atrophy after more than 12 months on GFD were anti-TG-positive. Silvester et al. (2017) conducted a meta-analysis to assess the sensitivity and specificity of

anti-tTG IgA and anti-endomysial antibody (EMA) IgA to identify CD patients with persistent villous atrophy despite GFD. They included 11 studies with a total of 1088 patients under GFD who underwent follow-up duodenum biopsy and serology. The sensitivity of anti-tTG IgA and EMA for villous atrophy was approximately 50%, which is very close to that found by Sharkey et al. (2013) in Cambridge. Therefore, serology appears to be a poor surrogate marker for mucosal recovery in GFD.

Therefore, monitoring of GFD adherence using the current tools (serology, symptoms, clinical interviews, and dietary surveys) has many limitations. Small bowel biopsy for the assessment of mucosal inflammation and villous atrophy is the "gold standard" for CD diagnosis. Mucosal healing would be an ideal parameter for monitoring GFD adherence and for clinical management. However, because of its invasiveness, relative risk, and cost, especially in asymptomatic patients, small bowel biopsy is not recommended in practice guidelines for monitoring disease activity and assessing dietary adherence in patients with CD, even when it is widely used by various clinical teams knowing the inaccuracy of the other monitoring methods (Sharkey et al. 2013). Thus, objective and non-invasive methods are needed to facilitate the monitoring of dietary adherence.

The validation of new, less invasive, and affordable diagnostic techniques could improve the identification of patients with CD who regularly transgress, either voluntarily or involuntarily, while following a GFD. This will improve adherence to the diet and maintain recovery from intestinal epithelial damage, thereby reducing the complications of an unsuitable GFD and differentiating between non-adherence and refractory disease. In this respect, monoclonal antibodies that can sensitively and specifically detect gluten immunogenic peptides (GIP) have been used in the development of immunoassays for the detection of inadvertent gluten consumption by measuring GIP in human samples such as stools and urine, thereby providing a potentiall tool for improving dietary adherence in patients with CD (Comino et al. 2012a, 2019; Moreno et al. 2017).

Development of a Method for the Detection of Gluten Immunogenic Peptides in Human Specimens

CD is triggered by the presence of peptides from the fragmentation of gluten, which are not digested by human proteases and are toxic to patients with CD. These peptides are called GIP and are resistant to gastrointestinal digestion and account for immunogenic reactions in the T cells of patients with CD. Shan et al. (2002) have shown in in vitro and in vivo studies in rats and humans that a 33-mer peptide from α-gliadin is stable against breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases. Furthermore, this peptide was identified as the primary initiator of the inflammatory response to gluten in patients with CD. The 33-mer peptide contains six partly overlapping, potentially harmful epitopes and is considered one of the most immunodominant gluten peptides for patients with CD.

To assess food toxicity, A1 and G12 monoclonal antibodies were generated against the α-2-gliadin 33-mer. These antibodies have been proven to be very useful

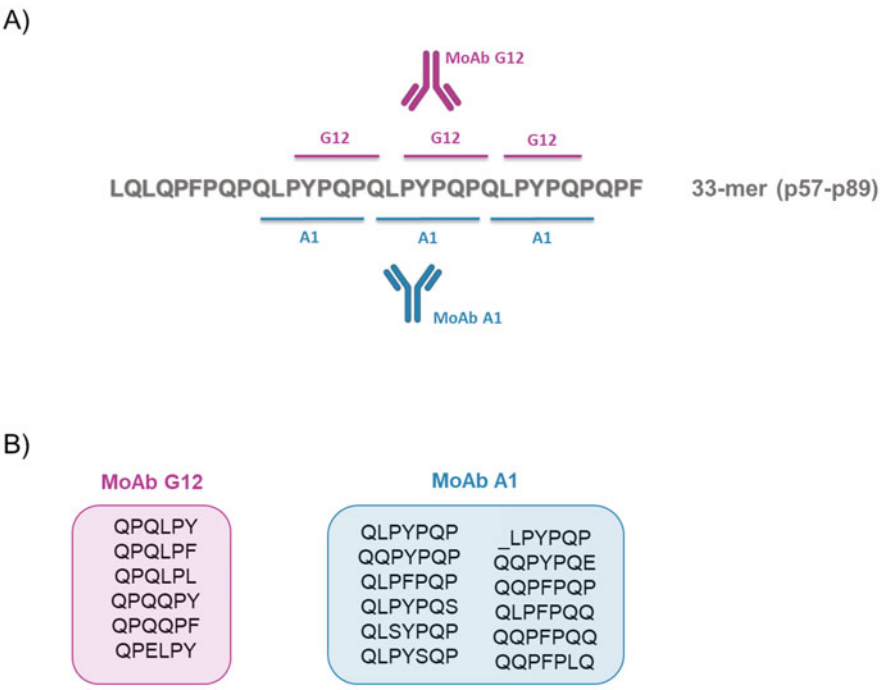

Fig. 1 Monoclonal antibodies G12 and A1 recognition regions in the 33-mer peptide. (**a**) Amino acid sequences of the α-2-gliadin 33-mer peptide. Epitope recognition of G12 and A1 monoclonal antibodies is indicated by a continuous line. (**b**) Recognition epitopes of G12 and A1 monoclonal antibodies. moAb, monoclonal antibody

for the detection of toxic peptides in food samples and for the enzymatic detoxification of gluten in clinical research (Morón et al. 2008a, b). It has also been demonstrated that these antibodies bind to most GIP in hydrolyzed food from different sources (Fig. 1). The 33-mer is resistant to hydrolysis via gastric, pancreatic, and intestinal brush border membrane proteases, which allows for its detection in human excretions (Camarca et al. 2009; Lindfors et al. 2019; Mitea et al. 2010; Moreno et al. 2016; Qiao et al. 2005; Shan et al. 2002). In addition, the anti-33-mer G12-based immunoassays showed that >30% of the gliadin-reactive peptides remained intact after hydrolysis during in vitro simulated gastrointestinal digestion (Comino et al. 2012a). Based on these findings, Comino et al. (2012a) described a novel method for monitoring GFD through GIP detection in human stools based on the use of the anti-gliadin 33-mer G12 antibody. Consequently, the recovery of measurable amounts of the immunotoxic fraction in stools indicates that gluten has passed through the digestive tract and that gluten therefore has been consumed. GIP was also detected in the stools of healthy individuals after consumption of a normal gluten-containing diet, a GFD combined with controlled ingestion of a fixed amount of gluten (Comino et al. 2012a). Moreover, GIP can be detected in urine samples 6–48 h after gluten intake (Moreno et al. 2017). The recovery of measurable amounts

of gluten peptides in urine indicates that gluten has been absorbed by the intestinal mucosa, has reached the circulation, and has been filtered by the kidney. Therefore, the A1 and G12 antibodies have been used in the development of immunoassays for the detection of gluten in food and for detecting significant amounts of excreted GIP in human fecal and urine samples (Comino et al. 2011, 2012a, b, 2013, 2016, 2019; Moreno et al. 2016, 2017; Morón et al. 2008a, b; Real et al. 2014).

The currently available tests for GIP detection in stool and/or urine are immunoassays adapted from those used for gluten detection in foods to maximize sensitivity. Lateral flow immunoassay (LFIA) tests can detect GIP from concentrations of 0.15 μg GIP/g in stools and 2.2 ng GIP/mL in urine after less than 30 min, showing a high sensitivity (98.5% and 97%, respectively) and specificity (100%) for both tests (iVYCHECK GIP Stool and iVYCHECK GIP Urine, Biomedal S.L., Seville, Spain) (Comino et al. 2012a, 2016; Ruiz-Carnicer et al. 2020). While these tests provide qualitative data, semiquantitative results could also be obtained from urine samples using the LFIA coupled with a lateral flow reader (iVYCHECK Reader, Biomedal S.L.), which has a quantification limit of 6.25 ng GIP/mL (Ruiz-Carnicer et al. 2020). Furthermore, the G12-based quantitative sandwich ELISA test for stool (iVYLISA GIP Stool kit, Biomedal S.L., Seville, Spain) allows for increased sensitivity and quantitative determinations. The analytical sensitivity of this test is 0.16 μg GIP/g stool, which is the limit of quantification, and it was validated in a multicenter clinical study, showing a diagnostic sensitivity and specificity of 98.5% and 100%, respectively (Comino et al. 2016).

Process to Consolidate the Detection of Immunogenic Peptides as a Biomarker in Patients with Celiac Disease

GIP detection can overcome some key unresolved scientific and clinical problems in CD management. GIP are resistant to gastrointestinal digestion and are responsible for immunogenic reactions in the T cells of patients with CD, and excreted GIP detection enables a direct and quantitative assessment of gluten exposure.

Several prospective studies have investigated the efficacy of GIP determination in stools and/or urine as a new non-invasive biomarker for detecting gluten intake and verifying GFD compliance in patients with CD (Comino et al. 2012a, 2016, 2019; Gerasimidis et al. 2018; Moreno et al. 2017; Porcelli et al. 2020; Roca et al. 2019; Silvester et al. 2020a, b).

Comino et al. (2012a) evaluated the capacity to determine gluten ingestion and to monitor GFD compliance in patients with CD by detecting GIP equivalents in stools. A total of 53 children with CD on GFD were enrolled. The participants were then exposed to a controlled ingestion of a fixed amount of gluten (9–30 g), and stool samples were analyzed using a G12 competitive ELISA. All the patients had detectable GIP on stools, ranging from 250 ppm (after exposure to 9 g) to more than 500 ppm (after exposure to 30 g). In a significant proportion of patients with CD, an abnormal small bowel morphology persisted despite a GFD, probably because of the persistent ingestion of trace amounts of gluten. Catassi et al. (2007)

showed that treatment of CD requires the ingestion of <50 mg gluten/day. To test whether the G12 antibody-based methods could detect small amounts of gluten in fecal samples, increasing doses of gluten (from 50 mg to a maximum of 1 g gluten/day) were administered after an initial 50 mg microdose. Gluten amounts in stools became detectable beyond the 50 mg dose of gluten. These findings demonstrated that the method is a reliable tool for the detection of GFD transgressions in children (Comino et al. 2012a).

Afterwards, a prospective multicenter study that included 188 patients with CD on a GFD and 73 healthy controls on a gluten-containing diet revealed that 56 patients (30%) with CD had high levels of GIP in their stools. All controls except one (98.5%) had quantifiable amounts of GIP in stool samples. The results for patients with CD also showed increasing dietary transgressions with advancing age (39% over 13 years) and sex (predominance of males in this evaluation). Simultaneously, the study also indicated the limitations of traditional methods, such as food questionnaires or serological tests, for monitoring a GFD in patients with CD (Comino et al. 2016).

Another prospective study on children with CD had similar results. GIP determination in stools of 44 children with CD on GFD and 19 newly diagnosed cases was performed in follow-up samples at 6 and 12 months after inclusion in the study. Compliance to GFD was evaluated based on clinical assessments, tissue transglutaminase (tTG) levels, and Biagi score, and the evaluations were compared to that of GIP detection. GIP was detectable in 16% of patients with a previous diagnosis of CD on GFD. In newly diagnosed patients, on a gluten-containing diet, GIP was detectable in 95% of the patients. Following GFD initiation, GIP decreased ($P < 0.001$), and 17% and 27% had detectable levels at 6 and 12 months, respectively. Compared to GIP, the Biagi score, tTG, and clinical assessment showed sensitivities of 17%, 42%, and 17%, respectively (Gerasimidis et al. 2018).

To investigate the course of gluten intake after a diagnosis of CD and subsequent GFD, the stools of 64 pediatric patients with CD were analyzed for GIP at diagnosis and 6, 12, and 24 months thereafter. Most children (97%) had detectable GIP at diagnosis. After GFD initiation, the rate of children with detectable GIP decreased to 13% at 6 months and increased to 25% at 24 months (Comino et al. 2019). A recent examination of 25 patients with CD on a GFD for at least 1 year revealed that 4 patients (16%) tested positive for stool GIP, 2 of which complied strictly with a GFD according to the Biagi questionnaire, and none of them manifested symptoms. The results demonstrated that stool GIP analysis identified patients who did not comply with a GFD more accurately than a validated questionnaire. Therefore, monitoring GIP in stools offers a direct and objective quantitative assessment of exposure to gluten after CD diagnosis (Porcelli et al. 2020).

Several prospective studies have been performed to investigate the efficacy of GIP detection in urine samples and, more importantly, its predictive value related to celiac histological damage. The first study made with urine samples included 76 healthy individuals aged 3–57 years (group 1) and 58 patients with CD aged 3–64 years (group 2) who were subjected to different dietary conditions. Subsequently, the kinetics or behavior in the elimination of GIP peptides in the urine of

healthy volunteers was studied. GIP were detectable in concentrated urine samples from group 1 individuals (previously subjected to a GFD) as early as 4–6 h after gluten intake (ingestion of at least a portion of pasta, bread, or whole grain from wheat, barley, and rye) and remained detectable for 1–2 days. The experiments indicated that the ingestion of >25 mg gluten could be detected in the urine. In group 2, patients showed a high percentage of gluten transgression. GIP in urine were also detectable in 48% of adults and 45% of children. Furthermore, an examination of duodenal biopsies from 25 adult patients with CD in group 2 revealed that most of the treated patients with CD who maintained mucosal healing (89%) had no detectable GIP in their urine, while all the patients with quantifiable GIP in their urine showed an incomplete intestinal mucosa recovery (Moreno et al. 2017).

Additional data were provided by a recent study, which supports the utility of GIP testing in CD management. The design of this prospective and controlled study with three groups of patients (healthy patients, CD patients with a de novo diagnosis, and patients with CD under GFD for more than 2 years), to whom it was performed simultaneously with celiac serology, dietary questionnaire (Celiac Dietary Adherence Test, CDAT), clinical interview, serial GIP determination in urine within 1 week (three urine samples, one on visit day and two during the weekend before the visit day), and duodenal biopsy, allowed a reliable correlation between the methods currently used for disease monitoring and urine GIP determinations versus duodenal histology as the gold standard. The authors found that 58% of the patients with CD consuming a GFD had detectable GIP in their urine at least once, with higher rates of positivity on the weekend. The results demonstrated a high sensitivity (94%) and negative predictive value (97%) of GIP measurements in relation to duodenal biopsy findings. In the GFD cohort, there was a statistically significant agreement between the absence of dietary gluten exposure, as detected by GIP concentrations and absence of histological lesions. In addition, the specificity of GIP measurements in predicting duodenal histological damage was 84.21% when the sample was collected on a workday or on Saturday. Additionally, they demonstrated that 25% of the patients on a GFD under follow-up for a median of 8 years presented with Marsh type II–III persistence of duodenal lesions, and 94% of them had GIP positive urine determinations. If serology, symptoms, or questionnaire scores were the only methods considered and that duodenal biopsy was not performed, 60–80% of these patients would have been overlooked. The potential future accumulated adverse effects in these patients with histological damage might not have been attributed to gluten consumption, and other alternative tests would have been unproductively prescribed to detect other etiologies. Thus, it was demonstrated that taking a GIP measurement on 3 days of the week, including the weekend, could be the best option to confirm GFD adherence in the short term and appears to accurately predict the absence of histological lesions (Ruiz Carnicer et al. 2020).

Furthermore, in patients with a de novo diagnosis who did not begin a GFD, the urine GIP concentrations were significantly higher than those in patients already consuming a GFD. However, in this group, four patients (18%) had concentrations below the quantification limit. Nonetheless, it is known that when facing with the least suspicion of CD and before completing the battery of diagnostic tests, many

patients eliminate or reduce gluten in their diet, making it difficult to reach an accurate diagnosis (Comino et al. 2016, 2019). Such behavior may result in the reduction of histological lesions at the time of diagnosis. These results suggest that during the diagnosis, measuring urine GIP concentrations could help in verifying whether a substantial amount of gluten had been previously ingested to validate the negative serological test results and the absence of histological damage (Ruiz Carnicer et al. 2020).

Applicability

Currently, there is no available method of monitoring adherence to GFD that ensures mucosal healing in patients with CD rather than biopsy. Intestinal biopsy is an expensive and aggressive follow-up method that is also not contemplated in clinical guidelines, although some recommend repeating intestinal biopsy one year after starting GFD. The measures that should be taken during follow-up are also unresolved; thus, many clinicians repeat duodenal biopsies throughout the follow-up of patients.

GIP determination in stools and urine has been demonstrated in numerous studies to be a direct and reliable method in detecting minimal amounts of gluten ingestion (Comino et al. 2012a, 2016, 2019; Gerasimidis et al. 2018; Moreno et al. 2017; Porcelli et al. 2020; Roca et al. 2019; Silvester et al. 2020a, b). The control of adherence to the GFD by determining GIP will allow to reveal dietary transgressions, which otherwise have been overlooked by clinical evaluation, adherence questionnaires, and serology. Furthermore, the identification of the transgressions would review the diet of patient and improve GFD adherence (Ruiz-Carnicer et al. 2020). Ensuring that a patient with CD does not resume the consumption of gluten, which may lead to asymptomatic intestinal damage, would eliminate the uncertainty in predicting the reappearance of villous atrophy and its possible complications and the association between any nonspecific symptomology and the underlying disease. In addition, concordan between the absence of excretion of GIP and normal duodenal histology point GIP determination as a biomarker of the mucosa status of patients. Having a biomarker of the state of the mucosa of patients with CD eliminates the uncertainty of the clinician during the follow-up and avoids the performance of intestinal biopsies that are aggressive and uncomfortable for the patient and expensive for the health systems.

A single GIP fecal test might be more sensitive than a single urine GIP test because the time window for GIP detection would be larger, and the possibility of the patient adhering to a GFD only for the medical visit could be avoided. However, most clinical laboratories and patients prefer to collect and handle urine samples, even if there are more samples to test. Furthermore, the determination of excreted GIP may provide a direct objective method in identifying low or no gluten consumption in the diagnosis of CD. It may also provide a method for the identification of potential false negatives in serology or in villus atrophy due to insufficient gluten intake by patients before their diagnosis is completed.

An additional problem is the assessment of the correct diagnosis of refractory CD, an infrequent disease that affects approximately 5% of patients with CD. Refractory CD diagnosis is established based on the exclusion of other disorders, persistence of malabsorption, and villous atrophy. Refractory CD also implies treatment based on adequate nutritional support and the use of corticosteroids and/or immunosuppressive agents, mainly azathioprine and infliximab. However, the main cause of the lack of response to a GFD is continued, usually inadvertent, gluten intake. Thus, it is especially important to accurately differentiate between refractory CD and inadvertent gluten intake, with villous atrophy persistence as a consequence. This is an evident application of GIP determination because the evidence of refractory CD implies the absence of elimination of GIP in stools or urine (Moreno et al. 2021).

Mini-dictionary of Terms

- **33-mer**: A peptide from α-gliadin that is stable against breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases.
- **Antibodies A1 and G12**: These antibodies have been used in the development of immunoassays for the detection of gluten in food and significant amounts of excreted GIP in human fecal and urine samples.
- **BIAGI**: A fast questionnaire based on four simple questions with a five-level score that verifies adult patient adherence with a GFD.
- **CDAT**: The Celiac Dietary Adherence Test is a clinically relevant, easily administered, 7-item tool for the standardized evaluation of GFD adherence.
- **GIP**: Gluten immunogenic peptides are resistant to gastrointestinal digestion and are responsible for immunogenic reactions in the T cells of patients with CD.
- **LFIA (antibodies G12 and A1)**: Lateral flow immunoassay tests can detect GIP in stool and urine samples.

Key Facts of Urinary Gluten Immunogenic Peptides as Biomarkers in Celiac Patients

Key Facts of GIP Determination in Human Specimens

- GIP are resistant to gastrointestinal digestion and are responsible for immunogenic reactions in the T cells of patients with CD.
- GIP determination is a non-invasive method that directly and quantitatively assesses gluten exposure.
- The low sensitivity of clinical symptoms, serology, and dietetic questionnaires in predicting GFD transgressions has been demonstrated in several studies with a poor concordance between positive GIP determination in urine or stools and other tools.

- Most patients with persistent villous atrophy have detectable GIP in urine and therefore ingest traces of gluten, but this consumption was not perceived by either symptomatology, serology, or dietary questionnaires.
- GIP determination in urine is a reliable biomarker of intestinal damage in patients with CD under GFD. In treated patients with CD, the absence of GIP in three urine samples on different days appeared to be the best procedure for predicting mucosal recovery in CD, as its negative predictive value was 97%.
- GIP determination in urine showed that patients with CD reduced gluten consumption before the physicians were able to complete their diagnosis.
- GIP determination is a useful tool for the differential diagnosis of inadvertent gluten ingestion in patients with persistent villous atrophy and refractory CD.

Summary Points

- CD is treated with a lifelong GFD.
- Current methods for monitoring GFD conformance, such as dietary questionnaires, serology tests, or clinical symptoms, may be inaccurate in detecting dietary transgressions, and duodenal biopsies are invasive, expensive, and not a routine monitoring technique.
- A promising advancement is the development of tests that measure GIP in stools and urine.
- Determining GIP concentrations in stool and/or urine samples is a highly sensitive and specific approach for assessing recent gluten exposure.
- Recent studies have shown that GIP determination in urine is a sensitive, clinically proven method for the follow-up of patients with CD, which may avoid serial invasive biopsies for determining gut mucosal recovery in patients with CD.
- Determining GIP concentrations in several urine samples could accurately predict the absence of histological lesions in patients with CD.
- The introduction of GIP testing as an assessment technique for GFD adherence may help in ascertaining dietary compliance and targeting the most suitable intervention during follow-up, avoiding the need for repeating duodenal biopsy and helping to differentiate symptoms related to CD and other conditions.

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Citrulline as a Marker of Villous Abnormality and Implications for Diet and Nutrition

43

Implications for Malabsorption and Nutrition

Alka Singh, Pooja, and Govind K. Makharia

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Abstract

Citrulline is a non-protein amino acid, which is synthesized from glutamine in the small intestinal enterocytes. It is involved in three important metabolic pathways, including the de novo synthesis of arginine in the kidney, intrahepatic transformation of ammonia to urea, and nitric oxide synthesis. Since citrulline is synthesized mainly by enterocytes, the blood levels of citrulline reflect the overall enterocyte mass. A reduction in the levels of citrulline reflects small intestinal enterocyte injury. Plasma concentration of citrulline has been considered to be a noninvasive marker of villous abnormalities and small intestinal length in short bowel syndrome. Plasma citrulline has also been explored to predict and assess the severity of rejection after small intestinal transplantation. Many inborn errors of metabolism such as deficiency of argininosuccinate synthase can lead to hypercitrullinemia and neurological dysfunctions. Since citrulline is a source of arginine and nitric oxide, its supplementation has been used in many cardiovascular conditions, sarcopenia, and erectile dysfunction. In this chapter, we have comprehensively discussed the synthesis and metabolism of citrulline as well as its diagnostic and therapeutic importance in human health.

Keywords

Citrulline · Enteropathy · Nitric oxide · Arginine · Biomarker · Nutrition · Celiac disease

Abbreviations

ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
CeD	Celiac disease
CPS	Carbamoyl phosphate synthase
CVID	Chronic variable immunodeficiency syndrome
g	Gram
GFD	Gluten-free diet
HSCT	Hematopoietic stem cell transplantation
L	Liter
NEC	Necrotizing enterocolitis
NO	Nitric oxide
NOS	Nitric oxide synthase
OAT	Ornithine aminotransferase
OCT	Ornithine carbamoyl transferase
OTC	Ornithine transcarbmoylase
P5CS	Pyrroline-5-carboxylate synthase
PBSCT	Peripheral blood stem cell transplantation
PPV	Positive predictive value
ROC	Receiver operating curve
SBS	Short bowel syndrome
μ	Micro

Introduction

Citrulline is a naturally occurring non-essential amino acid which is found in its free form in most biological fluids and synthesized in the small intestinal enterocytes from glutamine (Curis et al. 2005). Watermelon is a good dietary source of citrulline and in fact citrulline was first isolated from the watermelon juice (Rimando and Perkins-Veazie 2005). The synthesis and metabolism of citrulline involves the small intestine, liver, kidneys, and the endothelial cells through multistep enzymatic processes. Citrulline plays three important functions, namely, synthesis of arginine, synthesis of nitric oxide, and the intrahepatic transformation of ammonia to urea. As citrulline is converted into arginine, it is used as a precursor of nitric oxide and hence maintains the homeostasis of nitric oxide in the body (Windmueller and Spaeth 1981).

Since enterocytes are the site of synthesis of citrulline, concentration of circulating citrulline reflects the functional capacity of enterocytes and hence plasma concentration of citrulline has been proposed to be a noninvasive biomarker for detection of enteropathy. Crenn et al. in their first clinical study in the year 2000 have shown that plasma concentration of citrulline reflects small intestinal enterocyte mass (Crenn et al. 2000). Ever since many investigators have evaluated the value of plasma concentration in the assessment of enteropathies of various causes, post-chemotherapy mucositis, small intestinal transplant rejection, and neonatal necrotizing enterocolitis (Klipstein and Baker 1970; Dib et al. 2008; Adike et al. 2016; Das et al. 2016; Jeffers and Hourihane 1993; Malamut et al. 2010; Keefe et al. 2000; Singh et al. 2019; Singh et al. 2018).

There has been an increase in the interest of citrulline not only as a noninvasive marker of enteropathy or intestinal graft rejection but also of its therapeutic supplementation to increase muscle endurance, erectile dysfunction, and many other medical conditions (Singh et al. 2020; Gondolesi et al. 2002; Shiota et al. 2013; Bailey et al. 2015; Kim et al. 2015; Hess et al. 2017; Jirka et al. 2019; Suzuki et al. 2016). In this chapter, we have provided a comprehensive summary of the metabolic role of citrulline and its importance in diagnosis of diseases and its value as a nutritional supplementation.

Citrulline: Biochemical Characteristic, Synthesis, and Functions

Citrulline is a non-essential, non-protein amino acid and it is detectable in its free form in many biological fluids such as blood, urine, and cerebrospinal fluid (Curis et al. 2005). It was first isolated from watermelon in 1930; hence, it got its name from Citrullus vulgaris, the Latin name of watermelon (Rimando and Perkins-Veazie 2005). The richest dietary source of citrulline is watermelon white rind that surrounds the flesh has the highest concentration of citrulline. Other than watermelon, citrulline is also found in cucumber, muskmelon, bitter melons, and squash gourd. The concentration of citrulline in watermelon ranges between 0.7 and 3.6 g/kg of fresh weight (Rimando and Perkins-Veazie 2005). Since diet is the poor source of citrulline, endogenous synthesis is its main source/site.

The small intestine, liver, kidneys, and endothelial cells are involved in the production, metabolism, reabsorption, and turnover of citrulline (Windmueller and Spaeth 1981).

Synthesis of Citrulline from Glutamine in Enterocytes

While glutamine is considered as the main precursor of intestinal citrulline synthesis, other precursor amino acids such as arginine, proline, or ornithine can also be resourced from glutamine (Cynober et al. 1995; Wu et al. 1994). Supplementation of glutamine has been shown to increase intestinal citrulline and renal arginine synthesis and glutamine depletion from the diet has resulted in the lowering of plasma levels of citrulline in humans (Cynober et al. 1995; Morris 2000). The conversion of glutamine into the citrulline is a multistep enzymatic process where pyrroline-5-carboxylate synthase and ornithine carbamoyl transferase enzymes play a key role (Wu et al. 1994). Citrulline, following synthesis in enterocytes, is released across the basolateral membrane of enterocytes by ornithine-citrulline transporter into the portal venous circulation (Wu et al. 1994; Van De Poll et al. 2007; Maric et al. 2021). Unlike other amino acids, citrulline is poorly taken up by hepatocytes; therefore, large proportion of citrulline bypasses the liver and enters systemic circulation (Windmueller and Spaeth 1981).

Utilization of Citrulline in Different Tissues

There are three pathways that are associated with the metabolism of citrulline, depending on the tissue's distribution, namely, arginine biosynthesis, arginine-citrulline-nitric oxide cycle, and the urea cycle (Barzal et al. 2014) (Fig. 1).

Synthesis of Arginine from Citrulline

Arginine, a precursor of nitric oxide, plays an important role in regulation of intestinal blood flow, epithelial cell migration, and creatinine synthesis (Mori and Gotoh 2004; Morris 2000; Bode-Böger et al. 1998; Cynober et al. 1995). Hence, the conversion of citrulline into arginine is a very important factor for nitrogen homeostasis in the body. Arginine is considered as conditionally essential amino acid, which it is derived through endogenous synthesis and protein breakdown in addition to the dietary intake (Luiking et al. 2010). Arginine synthesized from citrulline represents 60% of total arginine synthesis (Morris 2000). Citrulline synthesized in enterocytes reaches the kidney through systemic circulation, where it gets converted into arginine by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) in the proximal tubule cells (Mori and Gotoh 2004) (Fig. 1). The amount of arginine synthesized from citrulline by the kidney is sufficient to fulfill the arginine requirement of the human body (Cynober et al. 1995).

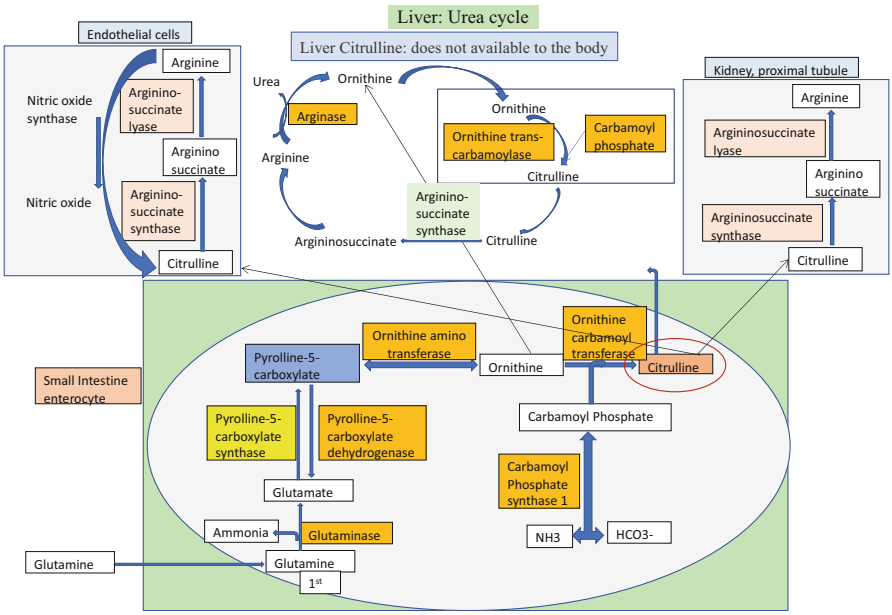

Fig. 1 Metabolic pathways of synthesis and utilization of citrulline in various metabolic processes. Citrulline is synthesized from the glutamine through a number of enzymatic reactions in the small intestinal enterocytes. Ornithine is also synthesized as an intermediate during citrulline synthesis in enterocytes. While ornithine is utilized in the urea cycle in the liver, citrulline plays an intermediary role in the urea synthesis in the liver. Most of the citrulline synthesized by enterocytes reaches the systemic circulation, mostly bypassing the liver. Citrulline is then converted into arginine by the argininosuccinate synthase and argininosuccinate lyase in the proximal tubular epithelial cells. Additionally, citrulline is converted into arginine and then arginine is converted into nitric oxide by nitric oxide synthase in the endothelial cell

Nitric Oxide Synthesis from Arginine and Citrulline

Since nitric oxide is synthesized from arginine and arginine from citrulline, it can be considered that citrulline plays a significant role in the synthesis of nitric oxide (Bode-Böger et al. 1998). Additionally, citrulline also plays an intermediary role during conversion of arginine to nitric oxide, catalyzed by the enzyme nitric oxide synthase (Bode-Böger et al. 1998). Nitric oxide plays important physiological roles in humans including vasodilation, endothelial functions, relaxation of various smooth muscles, neurotransmitter, inhibition of aggregation of platelets, prevention of leukocyte adhesion, and central regulation of blood pressure (Luiking et al. 2010; Morita et al. 2013). Nitric oxide has also been found to have anti-atherosclerotic effects (Luiking et al. 2010). Therefore, any dysfunction in the synthesis and availability of nitric oxide may cause endothelial dysfunction resulting in several cardiovascular diseases such as hypertensive atherosclerosis and angiogenesis-associated disorders (Luiking et al. 2012). Nitric oxide synthesis depends on the availability of arginine, which in turn depends upon the blood levels of citrulline (Morris 2000) (Fig. 1).

Urea Cycle

Endogenous nitrogen from enteral sources and muscle breakdown is excreted from the body as urea via urea cycle. Urea is synthesized in the liver from ammonia and carbon dioxide through a series of reactions using arginine and citrulline (Windmueller and Spaeth 1981). In hepatocytes, citrulline acts as an intermediary in the urea cycle, where it is converted by enzyme argininosuccinate synthase into argininosuccinate. Thus, citrulline utilized in the urea cycle is not released into the peripheral circulation (Haberle et al. 2012). As discussed above, citrulline synthesized in enterocytes and released in the portal circulation is not taken up by hepatocytes and bypasses the liver; therefore, citrulline metabolism in the liver is strictly compartmentalized (Van De Poll et al. 2007; Curis et al. 2005) (Fig. 1).

Normative Concentration of Citrulline in the Plasma

Normative concentration of citrulline in the plasma has been reported in few studies using very small number of healthy subjects. While studies have reported normal plasma citrulline concentration to be 40 ± 10 μMol/L, we in a study including 211 healthy volunteers in India found the normal plasma citrulline value to be 90.9 ± 51.2 μMol/L (Rabier and Kamoun 1995; Singh et al. 2020). We further observed that the concentration of citrulline does not change at 3 months, meaning thereby that the concentration of citrulline plasma remains stable. Based on the 10th percentile value of plasma citrulline concentration in healthy volunteers, a value of 39 μMol/L (95% confidence interval: 30.1–42.5 μMol/L) is considered normal.

Causes for High and Low Plasma Citrulline Levels

Since many enzymes are involved in the metabolism of citrulline, their deficiencies because of either inherited or acquired disease may lead to alterations in the plasma concentration of citrulline. Elevated plasma concentration of citrulline can be caused by deficiency of argininosuccinate synthase, which is an inborn error of disease called citrullinemia. High concentration of citrulline and hyperammonemia in them can lead to encephalopathy and early death (McMurray 1962; Ratner and Petrack 1951). Another inborn error deficiency of argininosuccinate lyase may cause accumulation of argininosuccinic acid with deficient endogenous arginine production and high levels of ammonia ultimately leading to neurocognitive decline (Allan et al. 1958).

Chronic kidney diseases may lead to lowered function of argininosuccinate synthase and argininosuccinate lyase activity and thus high level of citrulline in the plasma (Chen and Baylis 2010). Furthermore, high levels of citrulline have also been described after intestinal lengthening and after enterotrophic treatment with teduglutide (glucagon-like peptide 2), which increases intestinal mucosal growth (Dowling and Booth 1966; Jeppesen et al. 2012; Seidner et al. 2015).

While carbamoyl phosphate synthase 1 and ornithine transcarbamoylase can cause a lower concentration of plasma citrulline, the most common causes of low plasma citrulline are celiac disease, other enteropathic disorders, and short bowel syndrome (Funghini et al. 2012; Tuchman et al. 1989; Singh et al. 2020; Papadia et al. 2010; Malamut et al. 2010; Bailly-Botuha et al. 2009).

Diagnostic and Therapeutic Value of Citrulline

Based on its physiological functions, citrulline has been explored as a biomarker for the intestinal enterocyte mass and its function. As citrulline is synthesized almost exclusively by the small intestinal enterocytes, injury to these cells may cause a decline in citrulline concentration in the bloodstream as its synthesis is depleted. The declining plasma concentration of citrulline has been shown to correlate with enteropathy (Crenn et al. 2003; Oliverius et al. 2010). Therefore, the plasma level of citrulline has been assessed as a reflection of intestinal mucosal function in many small intestinal diseases such as short bowel syndrome, enteropathic diseases, necrotizing enterocolitis, chemotherapeutic drug-induced mucositis, and neonatal necrotizing enterocolitis (Adike et al. 2016; Malamut et al. 2010; Blijlevens et al. 2005; Jianfeng et al. 2005; Papadia et al. 2010; Herbers et al. 2010; Celik et al. 2013).

Furthermore, since citrulline is one of the building blocks of nitric oxide synthesis through arginine, functional supplementation of citrulline has been explored as a functional dietary supplementation in many conditions involving sarcopenia, cardiovascular diseases, and erectile dysfunction (Luiking et al. 2010; Nair 1995; Roubenoff and Castaneda 2001; Shiota et al. 2013; Cormio et al. 2011; Balderas-Munoz et al. 2012). In the following section, we shall discuss each of these two aspects of citrulline.

Blood Levels of Citrulline for the Assessment of Enterocyte Mass/Enteropathy

The main function of the gastrointestinal tract is the digestion and the absorption of the nutrients which is accomplished by the small intestine, while the rest of the part of gastrointestinal tract helps in the transit of food, regulated delivery of food to the small intestine, and excretion of the residuals (Mayhew 1999). Of the four layers of the small intestine, the small intestinal mucosa deals with both digestive and absorptive functions and other layers play a supportive role (Cheng and Leblond 1974). The main absorptive cells of the intestinal mucosa are enterocytes, which lines the small intestinal mucosal surface (Cheng and Leblond 1974; Das et al. 2016).

Enterocytes are very specialized cells, and they perform specific functions including absorption of nutrients and secretion of enzymes. As a consequence of its role in digestion, nutrient absorption, and waste excretion, the gastrointestinal tract is constantly exposed to harsh mechanical and chemical conditions (Mayhew 1999). Therefore, the intestinal tract has evolved mechanisms to cope with these assaults via a

highly regulated process of self-renewal (Lin and Barker 2011). Mucosal proliferation plays a fundamental role in the maintenance of the gut integrity (Mayhew 1999). Most of the epithelial cells are replaced every 3 to 5 days which is a high proliferation rate, second only to the hematopoietic system (Mayhew 1999). According to the so-called unitarian hypothesis, first proposed by Cheng and Leblond in 1974, this epithelial renewal is driven by a common intestinal stem cell (ISC) residing within the crypt base at the origin of the well-established crypt-to-villus hierarchical migratory pattern (Cheng and Leblond 1974). From their niche, intestinal stem cell gives rise to transit-amplifying cells that migrate upward and progressively lose their proliferative capability and maturate to become fully differentiated villous epithelial cells (absorptive enterocytes or secretory cells which include goblet cells, entero-endocrine cells, Paneth cells, and Tuft cells) (Barker et al. 2007). Each adult crypt harbors approximately 5 to 15 intestinal stem cells that are responsible for the daily production of about 300 cells; up to 10 crypts are necessary to replenish the epithelium of a single villus. Crypt-derived epithelial cells generally reach the villus tip after 3–5 days when they get apoptosed (Piscaglia et al. 2008; Das et al. 2016). These enterocytes can be damaged by a number of both acute and chronic diseases including celiac disease, tropical sprue, parasitosis, immunodeficiency syndromes, Crohn's disease, drugs such as olmesartan, etc. (Adike et al. 2016; Dib et al. 2008; Klipstein and Baker 1970; Malamut et al. 2010; Singh et al. 2019).

Plasma Citrulline as a Marker of Enteropathic or Reduction in the Absorptive Surface

The concept of using citrulline as a marker of small intestinal mucosal mass was proposed by Crenn et al., in 2000 (Crenn et al. 2000). They measured the plasma citrulline concentration in 57 patients with either permanent ($n = 37$) or transient ($n = 20$) intestinal failure. In patients with short bowel syndrome, citrulline levels were significantly lower than that compared with controls (20 ± 13μmol/L vs. 40 ± 10μmol/L, $p < 0.001$). On multivariable analysis, the level of citrulline correlated with the length of the residual small intestine. At a cut-off value of plasma citrulline of 20μmol/L, permanent intestinal failure could be differentiated from transient small intestinal failure with high sensitivity (92%), specificity (90%), positive predictive value (95%), and negative value (86%).

Crenn et al. continued their inquiry about the value of citrulline as a marker of enterocyte mass, and in 2003, they assessed the plasma citrulline levels in patients with villous atrophy due to celiac disease ($n = 42$), villous atrophy due to another cause ($n = 10$), healthy controls ($n = 51$), and severely malnourished patients with anorexia nervosa with no mucosal abnormalities ($n = 10$) (Crenn et al. 2003). Nine patients with celiac disease were revaluated after 1 year of gluten-free diet. Plasma citrulline concentration was found to be lower in patients with villous atrophy (both celiac and non-celiac) (24 ± 13μmol/L) than that in healthy controls (40 ± 10μmol/L) and patients with anorexia nervosa (39 ± 9μmol/L). Concentration of plasma citrulline was observed to be<10μmol/Lin patients with total villous atrophy, 10–20μmol/L with

proximal-only total villous atrophy, and 20–30μmol/L with partial villous atrophy. Levels of citrulline were also correlated with the severity and extent of villous atrophy ($r = 0.81$, $p < 0.001$). The ability of plasma citrulline to discriminate between patients with or without villous atrophy was also assessed using ROC curves, and plasma citrulline at concentration of 20μmol/L showed good sensitivity and specificity.

Later in 2005, Jianfeng et al. evaluated serum citrulline as a marker for small intestinal enterocyte mass and absorption function in patients with short bowel syndrome, where 22 patients with short bowel syndrome and 33 healthy controls were recruited (Jianfeng et al. 2005). Small bowel length and surface area were also assessed using contrast radiographs. Serum citrulline levels were found to be significantly lower in patients with short bowel syndrome in comparison to that in healthy subjects (5.9 ± 2.6 vs. 16.8 ± 5.9μmol/L). Six patients receiving rehabilitation therapy showed higher level of citrulline in comparison to that at the baseline, suggesting its role in the monitoring of patients during follow-up. The levels of plasma citrulline correlated with the length of remnant small intestine ($r = 0.82$, $p < 0.001$) and surface area ($r = 0.86$, $p < 0.001$). Citrulline level in the healthy Chinese control was observed to be much lower compared to studies in other populations. The lower level in healthy Chinese controls may be due to racial discrepancy or their long-term diet habits.

In order to assess the efficacy of plasma citrulline to predict villous abnormalities of modified Marsh grade 2 or more, we also recruited 131 treatment-naïve patients with CeD and 46 of them after 6 months of gluten-free diet, 216 healthy controls, and 131 patients with dyspepsia having no enteropathy (Singh et al. 2020). The median level of plasma citrulline was significantly lower in treatment-naïve patients with celiac disease compared to control [16.1 (IQR, 7.7–27.7) vs. 73.9 (IQR, 51.5–123), $p < 0.001$]. The plasma levels of citrulline also correlated with the grades of severity of villous abnormalities, plasma citrulline being lower with higher grade of villous abnormalities. In order to discriminate control subjects from patients with villous abnormality of modified Marsh grade 2, an optimum cut-off concentration values for plasma citrulline were found to be $\leq$30μmol/L. When a cut-off of plasma citrulline concentration of $\leq$30μM/L was considered, receiver operating curve (ROC) analysis showed a diagnostic accuracy of 89% with 78.6% sensitivity and 95.5% specificity, odds ratio of 77.9 (95% CI 31.6, 191), positive likelihood ratio of 17.4, and negative likelihood ratio of 0.22. The abovementioned statistics suggested that it is possible to predict significant villous abnormality based on plasma citrulline concentration even without obtaining duodenal mucosal biopsies in 78.6% of patients with 95.5% specificity in patients suspected to have celiac disease.

Cyclical Changes in the Level of Plasma Citrulline in a Model of Enteropathy

As discussed above, intestinal enterocytes are rapidly dividing cells and any injury to enterocytes leads to an increase in the replication from crypts (Cheng and Leblond 1974). These changes can be seen in those patients where rapid cyclical changes

occur in their enterocytes, such as patients receiving high-dose chemotherapy for hemopoietic stem cell transplantation (Blijlevens et al. 2005). This condition can act as a model of cyclical changes (normal enterocyte to injury to enterocytes and then recovery). If the blood levels of citrulline followed the cyclical changes as predicted in this model, such observation might support the efficacy of plasma citrulline as biomarkers in the prediction of the presence of enteropathy. Patients with hematological malignancy receiving high-dose chemotherapy for peripheral blood stem cell transplantation (PBSCT) could fulfill this criterion. We named this group as a model of predicted enteropathy.

In order to study the cyclical changes, we included patients receiving myeloablative therapy for hematopoietic stem cell transplantation ($n = 74$) (Singh et al. 2020). Heparinized plasma samples were collected at five time points, both before and after chemotherapy, and the concentration of citrulline was assessed. The concentration of citrulline at baseline was 117.3 ± 42.8μmol/L, which decreased and reached at nadir at day +7 following PBSCTs followed by progressive increase to reach at the baseline concentration at day +28 (131.9 ± 91.7μmol/L). As expected, we observed a cyclical pattern in the level of citrulline with the cycle of chemotherapy. The cyclical changes in the levels of citrulline were almost similar to the pattern of changes in the total leucocyte counts in the peripheral blood.

Citrulline and Mucositis

Mucositis is one of the effects of myeloablative therapy instituted before hematopoietic stem cell transplantation (HSCT). Mucositis affects not only the oral mucosa but also the gastrointestinal mucosa. While oral mucositis can be recognized easily with oral cavity examination, detection of intestinal mucosal injury requires endoscopic mucosal examination and histological assessment. In the year 2004, Blijlevens et al. assessed serum citrulline concentration serially on days −12, −6, 0, +7, +14, and +21 in 32 patients (mean age 48 years, range 25–65) receiving myeloablative therapy for HSCT transplantation (Blijlevens et al. 2005). They observed a decrease in the citrulline concentration till day +7 post-HSCT and then a serial increase in plasma citrulline concentration post-HSCT.

Herbers et al. also estimated citrulline concentration in 94 allogenic or autologous hemopoietic stem cell transplant recipients at baseline and at least once weekly after the start of myeloablative therapy until 30 days thereafter (Herbers et al. 2010). Their mean citrulline at the initiation of myeloablative regimen was 22.1 ± 8.1μmol/L which decreased significantly immediately after the start of myeloablative therapy and a nadir of plasma citrulline was observed around day 9.

Citrulline and HIV Enteropathy

In order to explore if the plasma concentration of citrulline correlates with the degree of reduction in the enterocyte mass in patients having tropical enteropathy, Papadia

et al. recruited 145 patients with tropical enteropathy, 44 of them having coexistent HIV infection. The level of citrulline was significantly lower in HIV-positive patients compared to HIV-negative ones (Papadia et al. 2010). Plasma citrulline concentration of 20μmol/L was able to discriminate between normal and damaged mucosa with 60% sensitivity, 59% specificity, 56% positive predictive value, and 63% negative predictive value.

Citrulline and Intestinal Transplantation Rejection

The diagnosis of rejection of intestinal transplant is made on the combination of clinical characteristics, endoscopic features, and histological characteristics (Pironi et al. 2015). Endoscopic examination and obtaining of biopsies for confirming intestinal rejection is considered invasive and cumbersome. A need for a noninvasive test, akin to serum creatinine for kidney transplantation rejection, for the assessment of intestinal graft rejection was felt (Pappas et al. 2002). Gondolesi et al. conducted a study to predict mucosal injury in patients having intestinal allografts (Gondolesi et al. 2002). They found a significantly lower concentration of citrulline in those having severe mucosal graft injury compared to those having no or mild graft injury (22.9 ± 15.4 vs. 38 ± 23.2 nmol/ml, $p < 0.001$). In another study, Pappas et al. included 26 patients undergoing small bowel transplantation and collected 387 blood samples (13 samples before transplantation and 374 post-transplantation) for the measurement of plasma citrulline (Pappas et al. 2004). The mean plasma citrulline concentration in pre-transplant samples was significantly lower in comparison to that in the controls (13.3 ± 11.5μmol/L vs. 34.8 ± 7.6μmol/L, $p < 0.001$). Assessment of plasma citrulline concentration in the post-transplantation period at different points of time showed that there was a more likelihood of a higher grade of transplant rejection if there was a slow increase in the plasma citrulline concentration toward normal level than when there was a rapid rise in plasma citrulline levels to >30μmol/L. A failure of the plasma citrulline concentration to return to normal levels was seen in patients who experienced more severe grade of graft rejection.

Citrulline in Patients with Necrotizing Enterocolitis

Necrotizing enterocolitis leads to impaired intestinal functions and it is one of the most common causes of morbidity and mortality in premature infants. In a study including 36 preterm infants (20 with necrotizing enterocolitis and 16 controls) with a gestational age of less than 30 weeks and birth weight of less than 1500 gm, the median citrulline concentration was significantly lower in infants with necrotizing enterocolitis compared to control (8.6μmol/L vs. 20.1μmol/L, $p < 0.05$) (Celik et al. 2013). In another study including17 preterm neonates with stage II necrotizing enterocolitis where serial plasma citrulline levels were assessed on days 2, 7, 14, 21, and 28, it was observed that the mean citrulline concentrations were significantly lower compared to controls on day 7, day 14, and day 21 (Ioannou

et al. 2008). In another study, levels of citrulline have been shown to decrease over 48 h after the onset, suggesting an increase in the intestinal injury during the first 48 h after the onset of necrotizing enterocolitis (Feenstra et al. 2021). El-Barbary et al. also reported a lower level of plasma citrulline in 40 preterm neonates with gestational age < 37 weeks with necrotizing enterocolitis in comparison to control (Nasr El-Din El-Barbary et al. 2018). While many studies have reported plasma citrulline in infants with necrotizing enterocolitis, most studies have included a smaller number of subjects; hence, a study with a larger number of patients is required to determine the most appropriate levels of plasma citrulline that is able to differentiate infants with necrotizing enterocolitis from healthy ones.

Levels of Citrulline as a Marker of Intestinal Function: A Meta-analysis

Fragkos et al. conducted a systematic review of 463 studies, of which 63 were included in the meta-analysis to report the efficacy of plasma citrulline in the prediction of small intestinal enterocyte mass and function in various small intestinal diseases including short bowel syndrome, enteropathies, post-chemotherapy, and HIV-induced enteropathy (Fragkos and Forbes 2018). They reported that citrulline levels correlate positively with small intestinal length in patients with short bowel syndrome ($r = 0.76$) and negatively correlate with the severity of intestinal disease such as celiac disease, tropical enteropathy, Crohn's disease, mucositis, and acute rejection in intestinal transplantation.

Therapeutic Role of Citrulline

Citrulline supplementation may aid in several health benefits. Oral citrulline supplementation boosts arginine plasma concentrations, enhancing its bioavailability as a substrate for nitric oxide generation, and thus exerts various health advantages (Table 1).

Muscles

Since citrulline plays an important role in the urea cycle, its supplementation has a potential to maintain ammonia homeostasis and removal of excess of ammonia from the circulation. Higher ammonia concentration enhances anaerobic glycolysis and lactic acid production in the muscles during high-intensity exercise and leads to muscular fatigue. Hence, it has been postulated that supplementation of citrulline can augment exercise performance either by improving skeletal muscle oxygen consumption resulting in better endurance or by lowering lactic acid production. A study including 56 obese and dynapenic human subjects has demonstrated an improvement in their upper limb muscle strength and walking speed with 10 g of citrulline

Table 1 Summary of evidence showing the efficacy/application of citrulline supplementation in prognosis, other diseases, or clinical conditions

Disease associated	Study design/ subjects	Citrulline dosage	Inference/results	Reference (PMID)
Muscle	Obese and dynapenic male and female ($n = 56$)	12-week high-intensity interval training +10 g dose of citrulline	Improved functional capacity and muscle function	Buckinx et al. (2018)
	Male cyclists ($n = 9$)	7-day supplementation of 6 g/day of citrulline	Improvement in time trial by 5.2%, significant increase in average heart rate, average rating of perceived exertion, and average power throughout the time trial	Stanelle et al. (2020)
	Old and young adults ($n = 26$)	7-day citrulline (6 g/day)	Improvement in the rate of rise in oxygen uptake at exercise onset in men	Ashley et al. (2018)
	Young men ($n = 41$)	8 g of citrulline malate 1 h before workout for 2 weeks	40% decrease in muscle soreness at 24 h and 48 h after the pectoral training session	Perez-Guisado and Jakeman (2010)
	Healthy adult male ($n = 10$)	Citrulline (6 g/day) for 7 days	Improvement in tolerance to severe-intensity exercise	Bailey et al. (2015)
	Sprague-Dawley male rats	5 g citrulline/kg/day for 1 week	Improved motor activity and maximal tetanic isometric force	Faure et al. (2012)
	Trained cyclists ($n = 22$)	2.4 g/day of L-citrulline orally for 7 days	Elevated plasma L-arginine levels and reduction in completion time by 1.5%. Better subjective feelings of muscle fatigue after exercise	Suzuki et al. (2016)
Cardiovascular diseases	Post-menopausal women ($n = 23$)	6 g/day of L-citrulline for 8 weeks	Decrease in sympathetic activity and blood pressure and increase in vagal tone	Wong et al. (2016)
	Healthy young and old heart failure adults ($n = 8$)	250 mg/100 ml of citrulline for the next 3 h (3 g)	Improvement in impaired nitric oxide synthesis in older heart failure patients	Kim et al. (2015)

(continued)

Table 1 (continued)

Disease associated	Study design/ subjects	Citrulline dosage	Inference/results	Reference (PMID)
	Patients with vasospastic angina	800 mg/day of L-citrulline for 8 weeks	Significant improvement in flow-mediated dilatation at 4 and 8 weeks as well as at 4 weeks after the end of intake	Morita et al. (2013)
	Healthy older adults ($n = 22$)	Nitrate-rich salad and citrulline drink for 1 month	Reduction in mean blood pressure, heart rate, and oxygen consumption during submaximal cycling and 5.2% increase in maximal power output	Le Roux-Mallouf et al. (2019)
	Children, undergoing cardiopulmonary bypass and at risk of pulmonary hypertension ($n = 40$)	5 perioperative doses (1.9 g/ $m^2$of citrulline per dose)	Postoperative pulmonary hypertension did not occur in children with citrulline elevations through supplementation	Smith et al. (2006)
	Young normotensive men ($n = 17$)	4 weeks of oral L-citrulline (6 g/ day)	Decrease in brachial systolic blood pressure, aortic systolic blood pressure, and aortic pulse pressure during cold pressor test	Figueroa et al. (2010)
	Stable outpatients ($n = 35$)	Oral L-citrulline supplementation (3 g/day) for 4 months	Increase in both left and right ventricular ejection both at rest and with stress	Balderas-Munoz et al. (2012)
Erectile dysfunction	Male patients with mild erection hardness score of 3 ($n = 24$)	L-citrulline, 1.5 g/day, for 1 month	12 (50%) 24 men when taking L-citrulline showed improvement in erection hardness score from 3 (mild ED) to 4 (normal erectile function)	Cormio et al. (2011)
	8-week-old male Wistar-ST rats	Oral 2% L-citrulline water supplementation for 3 weeks	Oral L-citrulline supplementation improved ICP/MAP and SM/collagen ratios and increased nitric oxide. Oral L-citrulline	Shiota et al. (2013)

(continued)

Table 1 (continued)

Disease associated	Study design/ subjects	Citrulline dosage	Inference/results	Reference (PMID)
			supplementation might be a useful novel therapy for acute arteriogenic erectile dysfunction	
	Male Wistar-ST rats	2% L-citrulline water (castrated + L-citrulline), 4 weeks	Improvement in erectile response to electric stimulation of cavernous nerve and penile structure in castrated rats	Hotta et al. (2014)
Depression	Patients with major depression and healthy controls	–	L-arginine and L-citrulline concentrations were significantly lower in patients with major depression in comparison to healthy controls	Hess et al. (2017)
Aging and sarcopenia	Male Sprague-Dawley rats	Citrulline 5 g/kg/ day for 1 week	Citrulline supplementation led to higher protein synthesis and protein content in the muscles	Osowska et al. (2006)
	Sprague-Dawley male rats	Citrulline 5 g/kg/ day for 1 week	Improvement in muscle mass and of motor activity	Faure et al. (2012)
	Healthy adults ($n = 8$)	11–24 g oral citrulline depending on the fat-free mass of the participant	Promotion of muscle protein synthesis	Jourdan et al. (2015)

supplementation along with 12 weeks of high-intensity interval training (Buckinx et al. 2018). In addition, citrulline supplementation (6 g) in trained cyclists ($n = 9$) resulted in the improvement in 40-km time trial by 5.2% along with a significant increase in heart rate, average rating of perceived exertion, and average power throughout the time trial (Stanelle et al. 2020). A similar study on trainee cyclists has also shown an increase in elevated plasma L-arginine levels and reduction in completion time by 1.5% along with improvement in subjective feelings of muscle fatigue on consumption of 2.4 g/day of L-citrulline orally for 7 days (Suzuki et al. 2016). Citrulline supplementation (6 g per day) has also been shown to increase oxygen consumption at the onset of exercise in young men (Ashley et al. 2018).

A randomized crossover study involving 10 healthy adult men has shown a reduction in mean arterial blood pressure, improvement in tolerance to severe-intensity exercise, and higher total amount of work completed in the exercise performance test with 7-day citrulline supplementation of 6 g/day (Bailey et al. 2015). Another study including 41 men, who performed 2 consecutive pectoral training session protocols, has shown a significant decrease in muscle soreness after the pectoral training session and a higher percentage response, implying better athletic performance during high-intensity anaerobic exercises with ingestion of 8 grams of citrulline malate (Perez-Guisado and Jakeman 2010).

Overall, the results of all the abovementioned studies show that citrulline improves skeletal muscle function and endurance.

Cardiovascular Diseases

Since citrulline along with nitric oxide leads to vasodilatation and maintains the vascular endothelium function, its deficiency has been linked with several cardiovascular diseases including hypertension, atherosclerosis, and diabetic angiopathy. Limited substrate availability of citrulline or arginine or the presence of inhibitors of these two amino acids may deplete circulatory nitric oxide levels. Citrulline is often used to elevate the levels of arginine which in turn augments synthesis of nitric oxide. Hence, improved physiological responses related to nitric oxide may be accomplished by citrulline supplementation.

An improvement in endothelial dysfunction (as assessed by flow-mediated dilatation) has been observed with supplementation of citrulline (800 mg/day for 8 weeks) in 22 individuals with vasospastic angina (Morita et al. 2013). Moreover, chronic supplementation of nitric oxide precursor (nitrate-rich salad and 6 g citrulline) has been reported to improve cardiorespiratory responses and lower resting blood pressure in 24 healthy older people (Le Roux-Mallouf et al. 2019).

Furthermore, oral citrulline supplementation has been effective in reducing postoperative pulmonary hypertension in children undergoing surgical procedures for congenital heart diseases (Smith et al. 2006). Similarly, a study involving 35 stable patients attending the Heart Failure Clinic has shown an improvement in left and right ventricular function both at rest and with stress with oral supplementation of 3 g/day of L-citrulline (Balderas-Munoz et al. 2012). In sedentary obese postmenopausal women, an 8-week supplementation of citrulline (6 g/day in two dosages) led to enhancement in cardiac autonomic function and resting heart rate variability and blood pressure (Wong et al. 2016).

Persistent administration of a combination of citrulline and arginine over 12 weeks in rabbits has been shown not only to reverse the effect of high cholesterol but also prevent the progression of atherosclerosis (Hayashi et al. 2005). A kinetic and vascular function study involving eight healthy young and eight adult patients with heart failure showed improvement in impaired nitric oxide synthesis in older heart failure adults with citrulline supplementation (Kim et al. 2015). In addition, young normotensive men ingesting oral L-citrulline (6 g/day) for 4 weeks in a

crossover design have also shown a decrease in systolic blood pressure, aortic systolic blood pressure, and aortic pulse pressure responses to cold pressor test (Figueroa et al. 2010). Some of this preliminary study suggests cardioprotective property of citrulline supplementation.

Erectile Dysfunction

Nitric oxide is a physiological signal essential for penile erection, since it acts not only as a neurotransmitter in the penile non-adrenergic, non-cholinergic nerve fibers but also as a vasodilator of the smooth muscles of the penile arteries, sinusoids, and trabeculae. Sexual stimulation releases nitric oxide in the smooth muscle of the penis. Oral citrulline supplementation has been shown to improve erectile function by increasing nitric oxide plasma concentration in a rat model with acute arteriogenic erectile dysfunction (Shiota et al. 2013). Furthermore, an improvement in the penile erection hardness score from 3 (mild erectile dysfunction) to 4 (normal erectile function) has been observed in 50% of 24 men taking 1.6 g of oral citrulline over a month, suggesting the effectiveness of citrulline in erectile dysfunction (Cormio et al. 2011).

Antidepressant Effects

Lower levels of arginine and citrulline have been reported to a higher risk of depression in a study of 35 healthy patients suffering from major depression (Hess et al. 2017). Therefore, it has been hypothesized that supplementation of citrulline and arginine may assist in the alleviation of depressive symptoms.

Ageing and Sarcopenia

An increase in muscle protein content and synthesis has been shown in elderly malnourished rats with the supplementation of citrulline at the dosage of 5 g/kg/day for 1 week (Osowska et al. 2006). A similar study in aged SD rats has also reported improvement in muscle mass and muscle motor activity with citrulline supplementation (Faure et al. 2012). An improvement in muscle protein synthesis has also been observed with oral ingestion of citrulline (11–24 g) in eight healthy adults on a low-protein diet (Jourdan et al. 2015).

Mini-Dictionary of Terms

Homeostasis: Tendency to maintain internal stability in an organism to compensate for environmental changes

Enterocytes: Absorptive intestinal epithelial cells lining the intestinal mucosa

Biomarker: A measurable indicator of the severity or presence of a disease state
Mucositis: Painful inflammation and ulceration of the mucous membranes lining the digestive tract
Endogenous: Growing or originating from within an organism
Precursor: Substance from which another substance is formed
Hepatocytes: Functional cells of the liver
Enteropathy: A disease of the intestine, especially the small intestine

Key Facts

- Citrulline is a ubiquitous amino acid, synthesized in the intestinal enterocytes from glutamine.
- Citrulline plays an important role in the production and regulation of arginine and nitric oxide.
- Plasma concentration of citrulline reflects the functioning enterocyte mass. A lower plasma concentration of citrulline suggests enteropathy.
- Since citrulline is a precursor of arginine and nitric oxide and helps in muscle synthesis, its supplementation has been explored as a therapeutic agent in various cardiovascular diseases, sarcopenia, and erectile dysfunction.

Summary Points

- Citrulline is a non-protein amino acid, and it is produced almost exclusively by enterocytes.
- Citrulline is a precursor of arginine and nitric oxide synthesis and it plays an intermediate role in the urea cycle.
- Since citrulline is synthesized mainly in enterocytes, the plasma levels of citrulline reflect the functioning enterocyte mass.
- The low concentration of citrulline in plasma (<30 microMol/L) reflects intestinal enterocyte damage and it may serve as a noninvasive biomarker of enteropathy.
- Plasma concentration of citrulline has been used for prediction and severity of graft dysfunction and graft rejection in patients undergoing small intestinal transplantation.
- Since citrulline is a precursor of arginine and nitric oxide and helps in muscle synthesis, its supplementation has been explored as a therapeutic agent in various cardiovascular diseases, sarcopenia, and erectile dysfunction.

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Biomarkers of Endothelial Dysfunction in Relation to Nutrition

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Abstract

Endothelial dysfunction is a term that covers diminished production and availability of nitric oxide as well as an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors. It is a leading cause of cardiovascular disease (CVD), and it can be considered a marker of risk and a prognostic marker.

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Several epidemiological studies have shown that adherence to a healthy diet pattern is associated with a reduction in the incidence of CVD and an improvement in plasma concentrations of biomarkers of endothelial dysfunction. This chapter reviews the evidence about endothelial biomarkers and their relationship to adherence to dietary patterns and specific nutrient intake.

Keywords

Endothelium · Endothelium markers · Endothelial dysfunction · Diet · Dietary patterns · Nutrients · Fatty acids · Phytochemicals

Abbreviations

A-II	angiotensin II
ALA	α-linolenic acid
ARA	arachidonic acid
COX	cyclooxygenases
CRP	C-reactive protein
CVD	cardiovascular disease
DHA	docosahexaenoic acid
EDN1	endothelin-1
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EV	extracellular vesicles
FMD	flow-mediated dilation
hs-CRP	high-sensitivity C-reactive protein
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
LA	linoleic acid
LDL	low-density lipoproteins
LOX	lipoxygenases
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
NF-κB	nuclear factor-κB
NO	nitric oxide
PUFAs	polyunsaturated fatty acids
ROS	reactive oxygen species
SELE	selectin E
TBARS	thiobarbituric acid reactive substances
TNF-α	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

Introduction

Many dietary compounds might be able to modulate and control the evolution of cardiovascular disease (CVD) such as liposoluble biomolecules closely related to cellular redox homeostasis and inflammation (e.g., fatty acids and polyphenols).

The endothelial system involves a number of potent vasoconstrictor peptides and their receptors, secreted by endothelial cells, such as EDN1 (endothelin-1) and eNOS (nitric oxide synthase). These molecules participate in the contraction and relaxation processes of vascular smooth muscle cells, among other functions. Other endothelial components, such as endothelial adhesion molecules, have been related to elevated blood pressure levels and in the pathogenesis of the atherosclerotic process. In this sense, classic and novel biomarkers are emerging for the study and prognosis of endothelial dysfunction (Leite et al. 2020).

Epidemiological and experimental studies indicate that various dietary factors are actively involved in modulating endothelial function (Defagó et al. 2013, 2014). Growing evidence indicates that healthy dietary patterns with a high intake of vegetables, legumes, fruits, nuts, whole grains, and unsaturated lipids may act offering protection against CVD and have been shown to be associated with some blood markers of endothelial function. On the other hand, Westernized food patterns with higher intake of processed food and rich in sodium, trans, and saturated fats are associated with worst endothelial function and risk of CVD. Regarding this, the chapter explores the main endothelial biomarkers related to specific dietary patterns and relevant nutrients in human studied.

Biomarkers: Clinical Endpoints

In the field of human health, the development, validation, and use of biomarkers as information tools to assess the risk factors associated with exposure to environmental agents increase daily. The term "biomarker or biological marker," refers to a broad subcategory of medical signs which can be measured accurately and reproducibly. It is an indicator of health status, life expectancy, or disease risk (Califf 2018). In this sense, a biomarker acts as an indicator of a normal biological process, a pathological process, or a response to a therapeutic intervention. At the same time, the measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction.

The evaluation of biomarkers in a biological sample and their interpretation in relation to the genesis and development of a disease are complex. However, they constitute an important tool to analyze the relationship between exposure and disease; to minimize adverse effects, giving greater precision to diagnosis and treatment; and to implement preventive strategies (Robb et al. 2016).

Classically, biomarkers can be grouped into three categories as follows (FDA-NIH Biomarker Working Group, 2016):

(a) *Exposure biomarker*: assesses in an organism the presence of an exogenous substance, a metabolite, or the product of the interaction between the xenobiotic agent and a target molecule or cell.
(b) *Effect biomarker*: assesses the biochemical, physiological, or behavioral alteration produced in the organism that can be associated with a pathological process.
(c) *Susceptibility biomarker*: indicator of the inherited or acquired capacity of an organism to respond to exposure to a xenobiotic substance (FDA-NIH). However, new categories have emerged with better specificity.
(d) *Diagnostic biomarker*: to detect or confirm the presence of a disease or condition of interest or to identify an individual with a subtype of the disease.
(e) *Monitoring biomarker:* to assess the status of a medical condition for evidence of exposure to a medical product or environmental agent or to detect an effect of a medical product or biological agent.
(f) *Pharmacodynamic/response biomarkers*: indicate the changes in response to exposure to a medical product or an environmental agent.
(g) *Predictive biomarker*: its presence or change may predict a favorable or unfavorable effect from the exposure to a medical product or environmental agent.
(h) *Prognostic biomarker*: to identify the likelihood of a clinical event, disease recurrence, or disease progression.
(i) *Safety biomarker*: to indicate the likelihood, presence, or extent of a toxicity as an adverse event due to an exposure to a medical intervention or environmental agent.
(j) *Susceptibility/risk biomarker*: indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition.

Nevertheless, categories may overlap on some occasions.

Endothelial Dysfunction

CVD, principally ischemic heart disease, and stroke are the leading causes of global mortality and a major contributor to disability. CVD is the primary consequence of metabolic syndrome and type 2 diabetes, and the main cardiovascular risk factors involved in its pathogenesis are abdominal obesity with increased visceral adipose tissue, hyperglycemia/insulin resistance, dyslipidemia, and endothelial dysfunction (Roth et al. 2020).

The endothelium plays a crucial role in maintaining the balance between the pro- and anticoagulation systems of the vasculature. It is an organ with a vital role in the regulation, maintenance, and control of cardiovascular functions. Any change that occurs in the endothelium by physical or chemical agents, with a molecular

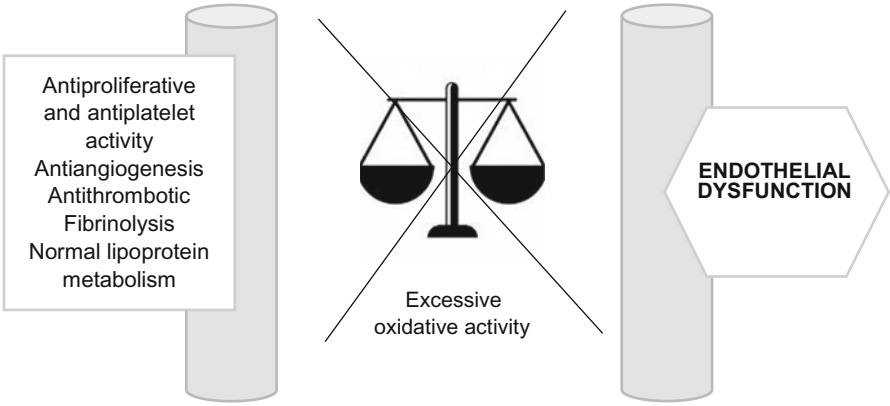

Fig. 1 Endothelial dysfunction

dysregulation in the metabolism of reactive oxygen species, leads to an alteration in its functions, predisposing it to platelet aggregation, thrombosis, inflammation, vasoconstriction, or an increase in vascular permeability. The *endothelial dysfunction*, leading to vascular damage in both metabolic and atherosclerotic diseases, is characterized by an abnormal proinflammatory and prothrombotic phenotype of the endothelial cells lining the lumen of blood vessels. As a consequence, there is a reduced nitric oxide (NO) bioavailability, impairment of the vascular tone, and other endothelial phenotypic changes (Godo and Shimokawa 2017) (Fig. 1).

In synthesis, endothelial dysfunction is characterized by a decreased bioavailability of antiatherogenic vasodilators and the consequent alteration of the homeostatic balance in favor of proatherogenic and prothrombotic vasoconstrictors, such as angiotensin II (A-II). The reduced endothelial capacity to maintain the medium homeostasis facilitates its permeability for the passage of lipids, promoting lipoprotein oxidation, inflammation, the proliferation of smooth muscle cells, the deposition or lysis of the extracellular matrix, platelet activation, and thrombogenesis. Platelet-derived mediators, such as serotonin, induce vasoconstriction in the presence of an activated or dysfunctional endothelium, and the vasoconstrictor response is magnified by the release of END1, a powerful vasoconstrictor (Förstermann et al. 2017).

Endothelial Function Assessment

Classical risk factors promote atherosclerosis development through mechanisms linked to endothelial damage. The measurement of endothelial dysfunction, one of the most important changes in the subclinical stage of atherosclerosis, can improve cardiovascular risk stratification and decisions regarding therapeutic interventions.

Due to the structure and diversity of endothelial functions, the measurement of endothelial dysfunction can be difficult. Although there is a wide range of techniques for identifying endothelial dysfunction that quantify different aspects of endothelial

Table 1 Noninvasive techniques for measuring endothelial function

Technique	Characteristics
Flow-mediated dilation	Measures the arterial dilation during post-occlusive reactive hyperemia using ultrasound imaging
Laser Doppler flowmetry	Measures the blood flow of skin microvasculature post-stimuli
Pulse wave velocity	Measures arterial stiffness
Finger plethysmography	Measures blood flow volume by recording changes in arterial pulsatile volume in the finger
Retinal endothelial function	Measures microvascular endothelial dysfunction by flicker light

physiology, the ideal measurement method is still uncertain today (Chia et al. 2020). In clinical practice, endothelial dysfunction can be evaluated by noninvasive techniques, although the use of these techniques is mainly applied for research purposes. The flow-mediated dilation (FMD) is measured by brachial artery ultrasound, retinal flicker test, and finger plethysmography – some of the methods of evaluating endothelial function of noninvasive approach (Alexander et al. 2021). All methods available for assessing endothelial function have certain limitations due to various factors, including variability of measurements by the operator; therefore, one technique is unlikely to be superior to the others. To overcome these limitations, the identification of endothelial biomarkers is crucial to complement current methodologies for timely endothelial dysfunction diagnosis. In recent decades, the study of endothelial dysfunction biomarkers and its clinical relevance have emerged as promising tools (Leite et al. 2020).

Table 1 summarizes the methods of evaluation of endothelial function noninvasive approaches and their main characteristics.

Biomarkers of Endothelial Dysfunction

Endothelial dysfunction is involved in the recruitment of inflammatory cells within the arterial intima and the initiation of the atherosclerotic process, for which the endothelium expresses cellular vascular (VCAM-1) and intercellular (ICAM-1) cell adhesion molecules, selectins (selectin E or SELE), and synthesizes and releases inflammatory cytokines and chemotactic proteins that promote the migration and penetration of monocytes and T-lymphocytes into the arterial wall. The adhesion of circulating lymphocytes to the vascular endothelium is the fundamental step for their extravasation during inflammation. Elevated values of VCAM-1, ICAM-1, and SELE have been observed in patients with coronary artery disease, which could indicate activation or damage of the endothelium as a particular component of a determined pathological process (Macías et al. 2003).

In this sense, although FMD is the most widely used method to study endothelial function, nowadays circulating biomarkers are a promising alternative. Numerous studies have been carried out evaluating classic endothelial circulating markers, such

as the aforementioned mentioned soluble adhesion molecules (SELE, ICAM-1, and VCAM-1); molecules involved in the cascade of coagulation, such as von Willebrand factor (vWF); and inflammatory markers, e.g., high-sensitivity C-reactive protein (hs-CRP) and the interleukins IL-6, IL-8, and IL-12. Recent evidence has proposed extracellular vesicles (EV) as possible biomarkers of endothelial dysfunction. These small vesicles (<1 mm) are released by the plasma membrane of various cells, such as endothelial cells and leukocytes, and contain biological materials from parental cells, such as adhesion molecules, substances involved in coagulation processes, and microRNAs that regulate gene expression (Di Pietro 2020). Besides, plasma endocan and endoglin levels have emerged as new cell dysfunction markers. Table 2 presents the main biomarkers related to endothelial dysfunction.

Biomarkers of Endothelial Dysfunction and Dietary Patterns

Cardiometabolic, behavioral, environmental, and social risk factors are major drivers of CVD. Currently, there is evidence that lifestyle, and specifically the diet, plays a fundamental role in the integrity and functionality of the vascular endothelium.

With advances in nutritional epidemiology during the last decades, the role of diet in the etiology of endothelial dysfunction is better understood. Several studies have shown that adherence to a healthy dietary pattern, characterized by a relatively high intake of fruits and vegetables, a source of phytochemicals and dietary fiber, has a beneficial impact on endothelial function, reflected through the decrease in circulating levels of adhesion molecules such as SELE, ICAM-1, and VCAM-1 as well as inflammation markers released into the circulation during endothelial injury. Vegetables contain bioactive compounds that act as exogenous antioxidant agents, inhibit and neutralize the activation mechanisms of free radicals, and protect tissues from the damaging effects of oxidative stress, a causative factor of endothelial dysfunction (Defagó et al. 2014).

It has also been observed that a diet rich in fish and seafood, a source of omega 3 polyunsaturated fatty acids, is associated with a decrease in the risk of coronary heart disease and cardiac events, mainly due to the anti-inflammatory and antiplatelet effects of their derived eicosanoids (Lovegrove and Griffin 2013). On the other hand, Westernized dietary patterns, characterized by a predominant consumption of red and processed meats, sweets, desserts, fried foods, and refined grains, with a predominance of saturated and trans fatty acids, cholesterol, simple sugars, and sodium, have been associated to an increase in the concentrations of inflammatory molecules and endothelial adhesion cells (Lopez-Garcia et al. 2004; Defagó et al. 2019). In particular, saturated fatty acids may increase plasma cholesterol, low-density lipoprotein, and triglyceride levels, mainly due to the fact that they decrease the hepatic uptake of LDL and favor the activity of the family of proteins that bind to the transcription factor of the sterol regulatory element binding protein (SREBP), with the consequent increase in this lipid fraction in the blood. Besides, trans fats also alter cholesterol and triglyceride levels and endothelial function; and

Table 2 Main classic and emerging biomarkers of endothelial dysfunction

Symbol	Biomarker name	Function
ICAM-1	Intercellular adhesion Molecule 1	Involved in leukocyte adhesion and inflammation. It is required for neutrophil migration into inflamed tissue
VCAM-1	Vascular cell adhesion molecule 1	Involved in the recruitment of leukocytes to sites of inflammation
EDN1	Endothelin 1	Acts as a modulator of vasomotor tone, cell proliferation, and hormone production. Works through its stimulating receptor, EDNRA (endothelin receptor A) and EDNRB (endothelin receptor B)
EDN2	Endothelin 2	Induces vasoconstriction, principally through EDNRA stimulation
NO	Nitric oxide	Is one of the endothelium-dependent relaxing factors released by the vascular endothelium and mediates vasodilation also inhibits platelet aggregation, induces disaggregation of aggregated platelets, and inhibits platelet adhesion to the vascular endothelium
AG2	Angiotensin II	A potent but labile vasoconstrictor produced from angiotensin I
DDAH1	Dimethylarginine dimethylaminohydrolase 1	Participates in NO generation by regulating cellular concentrations of methylarginines, which in turn inhibit NO synthase activity
SELE	Selectin E	Mediates adhesion and transmigration of leukocytes to vascular endothelium
EDNRA	Endothelin receptor type A	Participates in stimulation of cytokine release and endothelial growth factors
MEF2C	Myocyte enhancer factor 2C	Contributes to vascular endothelial growth factor (VEGF) expression in endothelial cells
SERPINE 1	Serpin peptidase inhibitor, clade E	Contributes to cardiac ventricular remodeling via migration of inflammatory cells and attenuation of extracellular matrix degradation
eNOS	Nitric oxide synthase 3, endothelial cell	Mediates the conversion of L-arginine in NO
VWF	von Willebrand factor	Has receptors for collagen, platelets, and ristocetin activity as well as the immunologically distinct antigenic determinants. Functions in adhesion of platelets to collagen and hemostatic plug formation. Marker of endothelial damage
EDNRB	Endothelin receptor type B	Participates in the control of vascular tone via stimulation of vascular smooth muscle cell receptors
CYBA	Cytochrome b-245, alpha polypeptide	Participates in the activation and stabilization of NADPH-oxidase
TGFB1	Transforming growth factor, beta 1	Regulates proliferation, differentiation, adhesion, migration, and other functions of the endothelial cell

(continued)

Table 2 (continued)

Symbol	Biomarker name	Function
COL18A1	Collagen, type XVIII, alpha 1	COL18A1 deficiency is associated with vascular endothelial cell damage and its degradation results in the generation of endostatin, a potent vasodilator
HM	Homocysteine	Induces endothelial dysfunction with respect to the regulation of vasomotor tone and hemostatic balance
FOL	Plasma folate	Participates in endogenous NO regeneration and bioavailability, and it has an antioxidant effect on vasculature
tPA	Tissue-type plasminogen activator antigen	Endothelial protein involved in thrombotic process as marker of baseline fibrinolytic capacity
FGB	Fibrinogen	Is a high molecular weight plasma adhesion protein and a biomarker of inflammation, related with hypertension development
SAA	Serum amyloid A	Impairs endothelium-dependent relaxation
Lp-PLA (2)	Lipoprotein-associated phospholipase A2	Potential link between noxious effects of oxidized LDL cholesterol, and marker of increased plaque vulnerability
Hs-CRP	High-sensitivity C-reactive protein	A plasma protein that circulates in increased amounts during inflammation and after tissue damage. CRP measured by more sensitive methods for coronary heart disease risk assessment is referred to as high-sensitivity C-reactive protein
TNF-αRII	Tumor necrosis factor-α receptor II	Induces proinflammatory cytokines, fibrin deposition, and an increase in permeability on endothelial cells
CP	C-peptide	Reduces endothelial cell surface expression of adhesion molecules. C-peptide may attenuate leukocyte-endothelium interaction. Activates and induces eNOS and the NO release from endothelial cells
IL-6	Interleukin-6	A cytokine that stimulates the growth and differentiation of B-lymphocytes and is also a growth factor for hybridomas and plasmacytomas. It is produced by many different cells including T-lymphocytes, monocytes, and fibroblast
IL-8	Interleukin-8	This cytokine plays a role in the regulation of acute inflammatory response. It is secreted by a variety of cell types and induces chemotaxis of neutrophils and other inflammatory cells
IL-12	Interleukin-12	It plays a role in innate and adaptive immune responses. It is produced by dendritic cells, macrophages, and other immune cells and plays a role in the stimulation of interferon-gamma production by T-lymphocytes and natural killer cells
END	Endostatin	Angiostatic proteins that are formed from proteolytic cleavage of collagen type XVIII

(continued)

Table 2 (continued)

Symbol	Biomarker name	Function
ENG	Endoglin	A membrane glycoprotein and angiogenesis factor that is expressed by cells of the vascular endothelium, vascular smooth muscle, and monocytes. It acts as a co-receptor for transforming growth factor beta and modulates cell adhesion
EDN	Endocan	Has the property of binding to a wide range of bioactive molecules associated with cellular signaling and adhesion and thus regulating proliferation, differentiation, migration, and adhesion of different cell types. An increase on its expression or levels is associated to endothelial activation and neovascularization
EV	Extracellular vesicles	Membrane-limited structures derived from cell membranes and cytoplasmic material, and released into extracellular space. They circulate through the extracellular fluid and through the peripheral blood in the microvasculature where bigger cells cannot, thereby affecting a variety of intercellular communication processes

their intake is associated with high levels of circulating inflammatory markers such as IL-6, ICAM-1, SELE, tumor necrosis factor, and hs-CRP (Mozaffarian and Clarke 2009). Specific functions of nutrients will be taken up later.

Table 3 summarizes the main findings in the relationship between dietary patterns and biomarkers of endothelial dysfunction.

Nutrients and Nutritional Components with Effect on Endothelial Dysfunction

Food patterns represent a real vision of the diet, including the combination of foods, as well as the possible synergy of different nutritional compounds (Morera et al. 2019). Clearly, the protective effect of healthy dietary patterns is conditioned by the variety of bioactive compounds and nutrients that jointly favor long-term cardiovascular health (Casas et al. 2018). Some of these individual nutrients have attracted the interest of numerous researchers, with the aim of exploring their effects on the modulation of endothelial dysfunction biomarkers, as well as the challenge of generating potential food sources and functional ingredients rich in antioxidant molecules.

To date, studies on phytochemicals, polyunsaturated fatty acids (PUFAs), and some vitamins and minerals show promising results. In clinical practice, the use of these natural components could constitute CVD complementary therapy because of their ability to attenuate mechanisms linked to oxidative stress, vascular inflammation, and endothelial dysfunction. Although there is strong evidence showing the

Table 3 Food patterns and endothelial system: summary data of main findings from epidemiological studies

Authors and publishing year	Food patterns	Principal results
Fung et al. (2001)	Prudent pattern (PP): Higher intakes of fruit, vegetables, whole grains, and poultry. Western pattern (WP): Higher intakes of red meats, high-fat dairy products, and refined grains	WP was positively correlated to fasting insulin, C-peptide, leptin, tPA, CRP, and homocysteine. PP was positively correlated with plasma folate and inversely correlated with insulin and homocysteine concentrations
Lopez-Garcia et al. (2004)	PP: Characterized by higher intakes of fruit, vegetables, legumes, fish, poultry, and whole grains. WP: Characterized by higher intakes of red and processed meats, sweets, desserts, French fries, and refined grains	WP was positively associated to CRP, SELE, ICAM-1, VCAM-1, and IL-6. PP was inversely associated with plasma concentrations of CRP and SELE
Esmaillzadeh et al. (2007)	Healthy pattern (HP): High in fruits, vegetables, tomato, poultry, legumes, tea, fruit juices, and whole grains. WP: High in refined grains, red meat, butter, processed meat, high-fat dairy, sweets and desserts, pizza, potato, eggs, hydrogenated fats, and soft drinks. Traditional pattern (TP): High in refined grains, potato, tea, whole grains, hydrogenated fats, legumes	HP was inversely associated to CRP, SELE, and VCAM-1. WP was positively related to CRP, SAA, IL-6, ICAM-1, and VCAM-1. TP without associations
Nanri et al. (2008)	HP: High intakes of vegetables, fruit, soy products, and fish. High-fat pattern (HFP): High intakes of fried food, meat, processed meat, mayonnaise, and egg. Seafood pattern (SP): High intakes of a variety of seafood, including shellfish, salted fish guts, fish roe, and fish-paste products. Westernized breakfast pattern (WBP): Principally bread, margarine, coffee, ham	HP was inversely related to CRP. HFP, SP, WBP: No associations were observed
Hlebowicz et al. (2011)	Low-fat and high-fiber pattern (LFHFP): Fruit, low-fat milk, both high-fat and low-fat meats, and sweet. Milk fat pattern (MFP): Bregott (a spread consisting of butter and rapeseed oil), cheese, whole milk, bread, and sweets. Fiber bread pattern (FBP): Fiber-rich bread, meats, sweets, fruits, low-fat	LFHFP was inversely associated to Lp-PLA (Andrukhova et al. 2014) activity. MFP and SCP were positively associated to Lp-PLA (Andrukhova et al. 2014). Other patterns: No associations were observed

(continued)

Table 3 (continued)

Authors and publishing year	Food patterns	Principal results
	margarine, and boiled potatoes. White bread pattern (WBP): White bread, low-fat margarine, both high-fat and low-fat meats and sweets. Many foods and drinks (FDP). Sweet and cake pattern (SCP): Sugar, sweets, jam, cakes, biscuits, and soft drinks	
Nanri et al. (2011)	HP: High in vegetables and fruit. Bread pattern (BP): High in bread and low in rice. Dessert pattern (DP): High in confections and fruit. Seafood pattern (SP): High in shellfish, squid, fish. WP: High in meat and fried foods	HP, BP, and DP were inversely associated to CRP. SP and WP were positively associated to CRP
Wood et al. (2014)	Prudent pattern (PP): High in the intakes of fish, yogurt, pulses, rice, pasta, and wine, in addition to fruit and vegetables. Meat-dominated dietary pattern (MDP): Meats, potatoes, fruit juice, and soft drinks. Processed food pattern (PFP): High intakes of cakes and confectionery. Past "detrimental" dietary pattern (PDDP): Dried/tinned fruit, soups and cereals. Contemporary "detrimental" dietary pattern (CDDP): Meat and potatoes	PP was negatively associated with serum hs-CRP concentration. MDP was associated with elevated serum hs-CRP concentration. Other patterns: No associations were observed
Poggio et al. (2017)	PP: Higher intake of fruits, vegetables, fish, seafood, whole cereal, and low-fat dairy products. WP: Higher intake of eggs, pastry and cakes, pizza, snacks, refined grains, red meat, vegetable oils, and poultry	PP was associated with reduced plasma concentrations of hs-CRP. WP was not significantly associated with any inflammatory biomarkers
Defagó et al. (2019)	Traditional dietary pattern (TDP): Higher intake of refined grains, red meat, whole fat dairy products, vegetable oils, and "mate," a traditional south American infused drink. PDP: Higher intake of vegetables, fruit, low-fat dairy products, whole grains, and legumes.	TDP adherence was associated with SELE Concentrations. PDP inversely associated with hs-CRP. Lower scores of adherence showed a positive relation with SELE. CDP was associated with SELE concentrations

(continued)

Table 3 (continued)

Authors and publishing year	Food patterns	Principal results
	Convenience and processed dietary pattern (CDP): Processed meat, snacks, pizza, and "empanadas," a stuffed pie served baked or fried	

potential cardiovascular health benefits of a great amount of dietary nutrients and bioactive compounds, it is important to note that supplementation with only one nutrient could not replace the effect resulting from the interaction between nutrients and nutritional compounds contained in a global dietary pattern; therefore, recommendations focused on healthy dietary habits, and not on individual foods or nutrients, are the cornerstone in the prevention and treatment of CVD.

Polyphenols

Phytochemicals are bioactive compounds of natural origin that exhibit a wide variety of biological effects with various benefits on human health. Within phytochemicals, polyphenols constitute a large and heterogeneous group with different capacities to modulate endothelial function with preventive effect on cardiovascular disease. Polyphenols are widely distributed in plant foods such as cocoa beans, nuts, olive oil, soybeans, sesame seeds, tea, red wine, vegetables, and fruits (Santhakumar et al. 2018).

Due to its antioxidant effect, in the last two decades, the study of polyphenols has increased considerably, arousing a remarkable interest among researchers. Although there are more than 8000 polyphenols, certain polyphenols have been studied in relation to CVD prevention by modulation of specific biomarkers (Di Pietro et al. 2020).

Currently, in vivo and in vitro studies have found convincing evidence of the therapeutic or preventive potential of these natural components. Epicatechin supplementation decreased SELE concentrates, while quercetin reduced SELE and IL-1β levels in prehypertensive men and women (Dower et al. 2015); fruit-based anthocyanins reduced CRP and IL-6 levels and increased FMD after a high-fat meal in overweight older adults (do Rosario et al. 2021); resveratrol, an important wine polyphenol, reduced vascular endothelial growth factor (VEGF), reactive oxygen species (ROS), IL-8, and ICAM-1 in an endothelial cell line (Toaldo et al. 2016). Other polyphenols such as chlorogenic acid, naringin, and epigallocatechin gallate have demonstrated beneficial results on endothelial function (Tsai et al. 2018; Malakul et al. 2018; Reddy et al. 2020).

While its ability to capture free radicals and control oxidative stress is known, polyphenols protect endothelial cells through various molecular mechanisms, beyond their antioxidant property. Polyphenols increase the activity of eNOS and NO production, decrease blood pressure and the lipid profile, inhibit gene expression of numerous proinflammatory signaling pathways, reduce adhesion molecules and proinflammatory cytokine concentrations, and modulate immune responses by improving interaction between immune cells (Di Pietro et al. 2020; Shakoor et al. 2021).

Clearly, plant polyphenols reduce the key events of endothelial dysfunction by providing benefits to combat numerous chronic diseases. Although the optimal dose and time of supplementation are not yet known, polyphenols may be a complementary alternative in conventional CVD therapy in the near future.

Polyunsaturated Fatty Acids

Fatty acids are dynamic molecules with the ability of modulating a wide range of cell signaling pathways, affecting endothelial function, inflammatory response, and the development of noncommunicable diseases (Defagó and Soria 2010). Dietary lipids are found in animal fats and plant oils and their effects on cardiovascular health depend on the nature of dietary fatty acids.

PUFAs have been widely studied in relation to CVD prevention. Within PUFAs, α-linolenic acid (ALA) and linoleic acid (LA) are the most abundant of the omega 6 and omega 3 families, respectively. LA is the precursor of arachidonic acid (ARA), while ALA is the precursor of *eicosapentaenoic acid (*EPA) and docosahexaenoic acid (DHA). These fatty acids are metabolized by cyclooxygenases (COX 1 and 2), lipoxygenases (LOX), cytochrome p450, or other subsequent enzymes or non-enzymatic reactions to form eicosanoids (Golanski et al. 2021). Eicosanoids are involved in various processes of vascular homeostasis. Scientific literature reports that eicosanoids derived from n-3 PUFAs have anti-inflammatory properties with benefits on endothelial cells compared with n-6 PUFAs. Diets rich in n-3 PUFAs, mainly derived from fatty fish intake, produce an increase in their concentrations in cell membranes, replacing AA. This leads to a lower production of eicosanoids derived from n-6 PUFAs and a substantial reduction in proinflammatory cytokine production, such as IL-1, IL-6, and tumor necrosis factor α (TNF-α) (Piper and Garelnabi 2020).

Other mechanisms of action of n-3 PUFAs in the regulation of vascular biomarkers and prevention of cardiovascular risk have been postulated and are known. DHA and EPA significantly attenuate the adhesion of monocytes, probably by decreasing the VCAM-1 and ICAM-1 expression and inhibiting NF-κB signaling pathway (Huang et al. 2015). n-3 PUFAs, mainly DHA, may have anticoagulation effects by suppressing platelet aggregation. Moreover, n-3 PUFAs promote vasodilation by stimulating NO production through eNOS gene expression in endothelial cells (Łacheta et al. 2019).

Antioxidant Vitamins

Many vitamins have a protective role on endothelial function; among them vitamins D, E, and C stand out.

Previous studies suggest that vitamin D deficiency is associated with an increase in endothelial dysfunction biomarkers and CVD predictors, including SELE, vWF, ICAM-1, VCAM-1, NO, and CRP (Oruc et al. 2017; Ilinčić et al. 2017). Vitamin D, in addition to its classic effects on calcium homeostasis, may act by modulating various mechanisms linked to endothelial injury (Kim et al. 2020). Endothelial cells have been shown to express vitamin D receptors and 1α-hydroxylase, and locally produced 1,25-dihydroxyvitamin D (Ashor et al. 2014), the active form of vitamin D, and they may act as an autocrine modulator of vascular disease (Zehnder et al. 2002). Vitamin D contributes to endothelial health by NO synthesis by mediating eNOS activity; decreases TNF-α, IL-6, VCAM-1, and ICAM-1 expression by suppressing NF-kB activation (Andrukhova et al. 2014; Rai and Agrawal 2017; Cimmino et al. 2020); and decreases ROS formation by increasing the expression of antioxidant enzymes and inhibiting the expression of NADPH oxidase (Dalan et al. 2014).

Vitamin C is found in numerous natural sources, including citrus fruits, kiwi, mango, strawberries, tomatoes, green leafy vegetables, and broccoli. Due to its antioxidant, anti-inflammatory, and immunomodulatory properties, ascorbic acid has been of particular interest in studying cardiovascular health. Data from clinical trials showed that vitamin C supplementation improves endothelial function, especially in people at higher cardiovascular risk (Ashor et al. 2014). Current evidence shows that 1 to 2 g per day of vitamin C is effective for both CRP reduction and endothelial function (Corrao et al. 2021). Vitamin C contributes to endothelium health through its antioxidant capacity, a role that has been repeatedly demonstrated in vitro. In addition, ascorbic acid stimulates endothelial cell proliferation through type IV collagen synthesis, prevents endothelial cell apoptosis, increases NO synthesis, prevents leukocytes from adhering to endothelial cells, and decreases thiobarbituric acid reactive substances (TBARS) as an oxidative stress index (May and Harrison 2013). A recent study using bioinformatics analyses provides evidence of anti-inflammatory effects of vitamin C mediated by JAK-STAT, STAT, PD1, EGFR, FoxO, and chemokine signaling pathways (Zhu et al. 2021). Although clinical and preclinical studies show protective cardiopropective of vitamin C, it is important to note that the benefits of vitamin C intake on CVD remain controversial. In future research, a key focus to elucidating the true effect of vitamin C will be to identify molecular mechanisms underlying the protective effects of vitamin C and therapeutic targets of endothelial damage.

Vitamin E is a potent antioxidant in lipid milieu, that is, it prevents the propagation of free radicals in membranes and in plasma lipoproteins. This vitamin is found in foods such as nuts, seeds, vegetable oils, leafy green vegetables, and fortified cereals. Vitamin E acts through antioxidant pathways and has been studied in relation to CVD, although to a lesser extent in relation to endothelial function. To date the results are contradictory. Some research that combines the effect of vitamin E

with other nutrients has shown cardiovascular health benefits. In vitro studies have shown that vitamin C and E supplementation has synergistic antioxidant effects, which could be explained by the concept of "vitamin E recycling," where tocopherol radical (Vit E-O•) is restored by vitamin C (Traber and Stevens 2011).

Antioxidant Minerals

Selenium, magnesium, and zinc are essential for protecting cells from oxidative damage. Selenium is a key component of glutathione peroxidase and thioredoxin reductase, enzymes that prevent vascular oxidative stress and endothelial dysfunction. Clinical studies show a relationship between adhesion molecules and selenoproteins as biomarkers of selenium status (Lopes Junior et al. 2019). Even in inflammation conditions, selenium needs increase (Manzanares et al. 2009). Selenium deficiency has also been reported in patients with different inflammatory-based diseases including stroke, diabetes, and atherosclerosis, but the pathophysiological mechanisms explaining the effect of selenium on endothelial dysfunction remain unknown.

Clinical study findings show that magnesium supplementation increases FMD in adult subjects. Magnesium, an abundant mineral in the body, participates in physiological mechanisms that are inherent to vascular endothelium; among them, it promotes the production of vasodilator molecules, regulates collagen and elastin in the vascular wall, maintains the elasticity of the vessels, regulates vascular tone, and modulates blood pressure. Magnesium is found in many foods, including whole grains. Due to processing or refinement of various foods, Western diets provide low magnesium content. Inadequate intake of this mineral is associated with the development of insulin resistance, hyperglycemia, and altered lipid metabolism, processes that increase atherosclerotic changes, arterial stiffness, and CVD development. In addition, magnesium deficiency increases the inflammatory response and circulating levels of cytokines with a negative impact on the vascular endothelium (Marques et al. 2020).

Zinc is an oligoelement that participates as a cofactor in the metabolism of numerous molecules. In addition, zinc exerts antioxidant properties. Available evidence shows that zinc is necessary for the dimerization of eNOS and subsequent NO production. It has also been documented that zinc has anti-inflammatory properties, so its deficiency may increase the predisposition to vascular inflammation. In an experimental model zinc deficiency increased IL-1β generation in human mononuclear cells after stimulation with LPS; in contrast, zinc supplementation decreases hs-PCR, IL-6, monocyte chemoattractant protein-1 (MCP-1), SELE, and VCAM-1 (Prasad 2014). Other benefits of zinc supplementation have been observed in the prevention of risk factors associated with endothelial dysfunction, including metabolic syndrome, dyslipidemia, and blood pressure (Zalewski et al. 2019).

Figure 2 summarizes the effect of dietary patterns and their bioactive compounds on the underlying mechanisms of endothelial dysfunction.

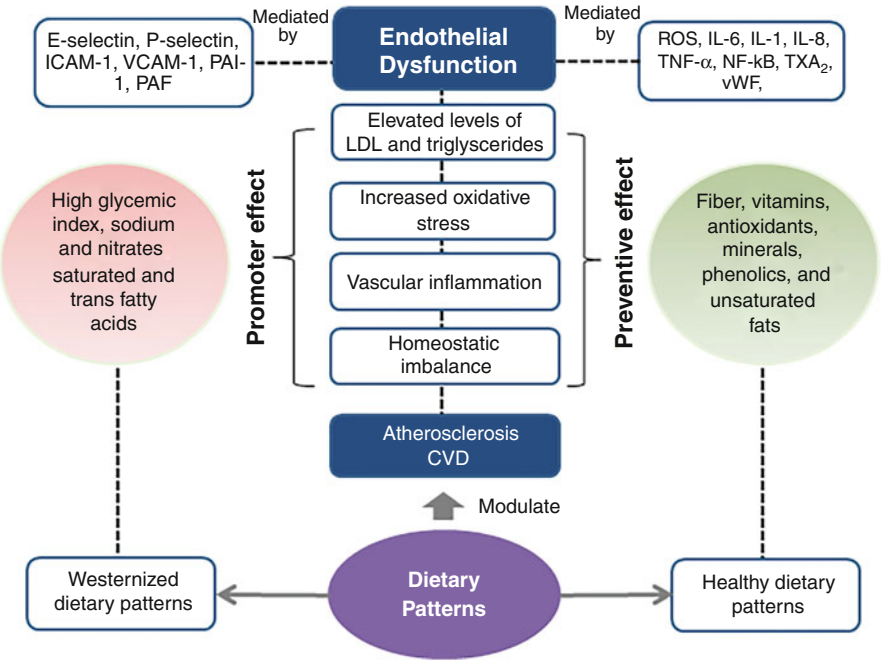

Fig. 2 Effect of nutrition on endothelial dysfunction. VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular adhesion molecule; PAI-1: plasminogen activator inhibitor-1; PAF: platelet-activating factor; ROS: reactive oxygen species; IL-6: interleukin-6; IL-1: interleukin-1; IL-8: interleukin-8; TNF-α: tumor necrosis factor α; NF-κB: nuclear factor-kB; vWF: von Willebrand factor; TXA_2: thromboxane A_2

Applications to Prognosis

Diet and nutritional molecules can play an important protective role in cardiovascular health. However, it is necessary to translate the research results to clinical practice, for example, including the measurement of endothelial adhesion molecules and hs-CRP in routine tests, as well as triangulating laboratory results with variables associated with lifestyle, such as diet (Defagó et al. 2014).

Mini-Dictionary of Terms

Bioactive compounds: nutritional constituents that are found in small quantities in foods providing health benefits beyond the basic nutritional value of the product.

Dietary pattern: is defined as the quantity, variety, or combination of different foods and beverage in a diet and the frequency with which they are habitually consumed.

Endothelial dysfunction: alterations in endothelium regulating functions, resulting in imbalanced production of relaxing and contracting factors, pro-coagulant and anticoagulant mediators, or growth-inhibiting and growth-promoting substances.

Oxidative stress: phenomenon caused by an imbalance between production and accumulation of oxygen reactive species in cells and tissues and the ability of a biological system to detoxify these reactive products.

Key Facts of Endothelial Dysfunction and Nutrition

- Diet is a factor liable to be modified, which offers an opportunity for prevention and treatment of CVD.
- Scientific and health societies support the consumption of healthy eating patterns, such as Mediterranean, Prudent, or DASH diet (Dietary Approaches to Stop Hypertension), for the prevention of cardiovascular diseases.

Summary Points

- The endothelium is an organ with a vital role in the regulation, maintenance, and control of cardiovascular functions.
- Circulating biomarkers are a promising alternative to study endothelial function.
- Dietary compounds might be able to modulate and control the evolution of CVD.
- Healthy dietary patterns with a high intake of vegetables, legumes, fruits, nuts, whole grains, and unsaturated lipids may act offering protection against CVD and have been shown to be associated with some blood markers of endothelial function.
- Westernized food patterns with higher intake of processed food and those rich in sodium, trans, and saturated fats are associated with worst endothelial function and risk of CVD.
- Bioactive compounds such as phytochemicals, polyunsaturated fatty acids, and some vitamins and minerals show promising results in dietary supplementation to attenuate inflammation and oxidation mechanisms.

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Creatine Kinase as a Biomarker

45

Links with Statins, Muscle, and the Response to Diet – A Focus on the Use of Nuts

Lígia Moriguchi Watanabe, Marcela Augusta de Souza Pinhel, Natália Yumi Noronha, and Carla Barbosa Nonino

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Abstract

Creatine kinase (CK) has been widely used as a biomarker in clinical practice to identify muscle disorders and other health conditions. Studies about its structures and functions have advanced over time and provided important tools for elucidating mechanisms related to muscle metabolism and the pathologies associated with disturbances in these mechanisms. One of the most expressive uses of CK is

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identifying and treating statin-associated muscle symptoms (SAMS), the most studied adverse effects of statins associated with nonadherence to treatment and unfavorable cardiovascular outcomes. The single-nucleotide polymorphisms (SNPs) in genes related to statin metabolism pathways can be highlighted among the risk factors that predispose to SAMS. Identifying these SNPs in patients proves to be a tool for health professionals in prescribing statins and adjusting their dosage. Nut consumption has been widely associated with health benefits, especially chronic diseases. In this sense, some studies suggest using nuts as adjuvants to drug therapies, improving health parameters such as lipid profile, oxidative stress, and the decrease in serum CK levels. In this context, the consumption of nuts may assist in statin therapy and possibly mitigating the SAMS, although more studies are needed to elucidate the involved mechanisms. Finally, there is recent evidence interconnecting the CK levels, SAMS, and nut consumption.

Keywords

Creatine kinase · Muscle disorders · Statin · Statin side effects · Statin-associated muscle symptoms · Single-nucleotide polymorphisms · Variability · Nut consumption · Nuts health benefits

Abbreviations

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette subfamily B member 1
ABCG2	ATP-binding cassette subfamily G member 2
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
CK	Creatine kinase
CoQ10	Coenzyme Q10
Cr	Creatine
CYP2C9	Cytochrome P450 family 2 subfamily C member 9
CYP3A4	Cytochrome P450 family 3 subfamily A member 4
CYP450	Cytochrome P450
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvic transaminase
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	Hydroxy-methyl-glutaryl coenzyme A
IFCC	International Federation of Clinical Chemistry
LDH	Lactic dehydrogenase
LDL-C	Low-density lipoprotein cholesterol
Mt-CK	Mitochondrial creatine kinase
MUFA	Monounsaturated fatty acid
OATP1B1	Organic anion transport protein 1B1
PCr	Phosphocreatine

P-gp	P-Glycoprotein 1
PUFA	Polyunsaturated fatty acid
SAMS	Statin-associated muscle symptoms
SLCO1B1	Solute carrier organic anion transporter family member 1B1
sMtCK	Sarcomeric mitochondrial creatine kinase
SNPs	Single-nucleotide polymorphisms
uMtCK	Ubiquitous mitochondrial creatine kinase

Introduction

Creatine kinase (CK), also known as creatine phosphokinase, is a dimeric enzyme generally found in all vertebrates (Sumien et al. 2018). By reversible interconversion of creatine (Cr) into phosphocreatine (PCr) (Fig. 1), named CK/PCr system, CK builds up a large pool of rapidly diffusing PCr that is available as a temporal or spatial energy buffer in cells of large and fluctuating energy demands, such as the

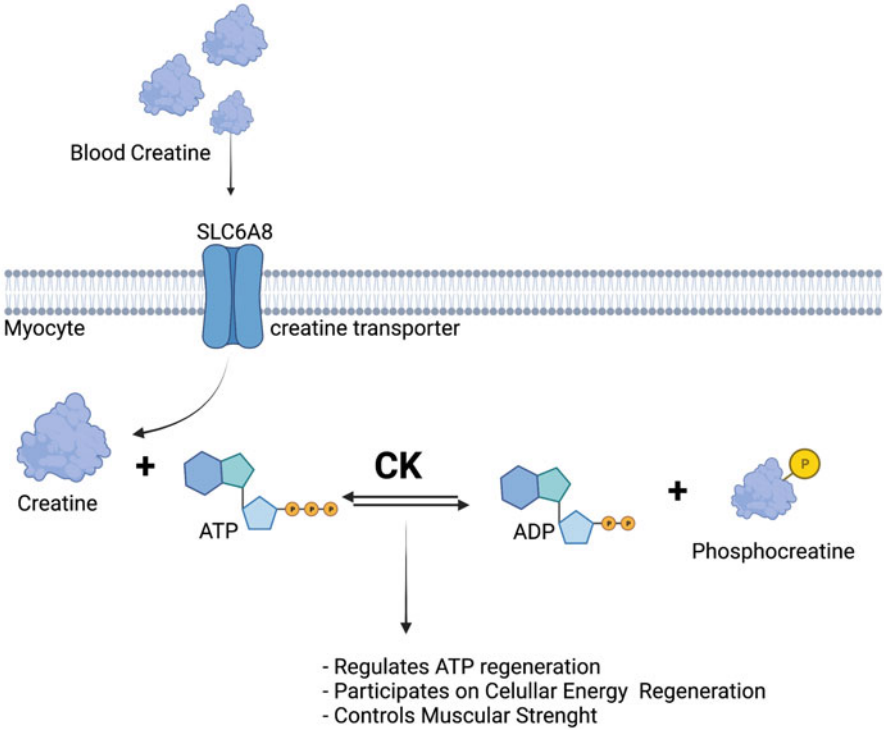

Fig. 1 Creatine kinase (CK)/phosphocreatine system. CK catalyzes the conversion of creatine and uses adenosine triphosphate (ATP) to create phosphocreatine and adenosine diphosphate (ADP). This reaction is reversible, and ATP can be generated from phosphocreatine and ADP. (Created with BioRender.com)

heart, skeletal muscle, and brain (Saks et al. 1996; Schlattner et al. 2006; Wallimann et al. 2011). CK/PCr system also prevents the rise in intracellular free adenosine diphosphate (ADP) by regulating the net pool of adenine nucleotide in the cell, acts as a proton buffering system, and stimulates oxidative phosphorylation in the mitochondria and adenosine triphosphate (ATP) consumption in the cytosol (Sumien et al. 2018).

Since its discovery in the early 1930s, serum CK concentration is increasingly used to assess muscular diseases due to its higher muscle specificity and greater sensitivity (Vassella et al. 1965). Besides pathological conditions, CK levels in the plasma can be altered by numerous factors, including sex, ethnicity, age, physical activity, and medications (Sumien et al. 2018). Statin-associated muscle symptom (SAMS) is the most well-documented side effect of statins and is the primary reason for discontinuation and nonadherence of statin therapy, remaining a gap in both the primary and secondary prevention of atherosclerotic cardiovascular disease (Stroes et al. 2015; Thompson et al. 2016; Ward et al. 2019). SAMS cover a broader range of clinical presentation and can present as myalgia, myopathy, myositis with elevated CK, or, at its most severe, rhabdomyolysis (Ward et al. 2019). Understanding variation in CK levels is vital for appropriate administration of statin therapy to all population segments to identify individuals at increased risk of developing side effects and provide alternative treatment strategies (Neal et al. 2009; Ward et al. 2019).

The elucidation of the contributing factors to statin response variability may prove to be an essential public health accomplishment (Zineh 2007). Although these factors include social and environmental influences, there has been an interest in investigating genetic variants' contribution to variability in the drug response (Zineh 2007). Several studies have reported that polymorphisms in genes encoding drug-metabolizing enzymes influence the liver metabolism of specific statins and can cause a relevant effect on therapeutic response and the risk of adverse effects, including SAMS (Hirata et al. 2018).

Including foods with antioxidant characteristics may be a worthwhile strategy to improve antioxidant capacity and reduce SAMS risk in patients using statins (Watanabe et al. 2021). There is increasing interest in nut consumption and beneficial health effects on metabolic risk factors such as oxidative stress, inflammation, visceral adiposity, hyperglycemia, insulin resistance, endothelial dysfunction, and metabolic syndrome (Coates et al. 2018; de Souza et al. 2017). The health benefits of nuts are attributed to their greater composition of healthy fatty acids, vegetable proteins, fibers, vitamins, minerals, carotenoids, and phytosterols (Cardoso et al. 2017). Accordingly, the nut consumption could be an adjuvant in statin therapy, contributing to the lipid profile and potential antioxidant action, reducing SAMS risk. More recently, nut consumption was associated with lower levels of CK in patients using statins with and without SAMS, and although the mechanisms had not been elucidated, the decrease in CK was connected to lower oxidative stress (Watanabe et al. 2020). Nonetheless, the current findings of the benefits of nut consumption on human health have not yet been discussed, and further studies should be carried out to evaluate the effect of nuts on specific pathologies, such as SAMS.

Creatine Kinase Historical Overview: Interrelationships with Skeletal Muscle Disorders

Creatine kinase (CK) reaction was first identified by Karl Lohman in 1934 while studying the chemistry of muscular contraction (Bessman and Carpenter 1985; McLeish and Kenyon 2005). CK is crucially involved in many bioenergetic processes and is expressed at high levels in cells with high energy requirements (Wallimann et al. 1998). The most important feature for the cellular functions of the CK/PCr system is the presence of tissue- and cell-specific CK isoforms with defined subcellular locations (Wallimann et al. 2011). Since the CK isoenzymes discovery in the early 1960s, new studies were carried out, generating discoveries about their chemical characteristics, functions, distinct forms, and subunits (Dawson et al. 1965; Eppenberger et al. 1967; Jacobs et al. 1964). Cytosolic CK comprises two polypeptide subunits of 42 kDa units each, designated M and B (Panteghini 1988; Sumien et al. 2018; Wallimann et al. 2011). These subunits come together to form dimers that are present in three tissue-specific isoenzymes: MM-CK, the major isoform in the muscle (98%) and heart (70–80%); MB-CK, mainly present in the myocardium (20–30%); and BB-CK that exists in many tissues, especially the brain (Sumien et al. 2018; Wallimann et al. 2011). Another form of CK is localized in the outer mitochondrial compartment, between mitochondria's cristae and intermembrane space (Mt-CK). The two different isoenzymes of Mt-CK are sarcomeric MtCK or sMtCK, expressed mainly in the cardiac and skeletal muscle, and ubiquitous MtCK or uMtCK, expressed in the brain, smooth muscle, and sperm (Sumien et al. 2018; Wallimann et al. 2011). The Mt-CK complex is expressed as an octameric structure to form a functional entity (Sumien et al. 2018).

The different characteristics and expression patterns of the CK isozymes account for the cell-compartmentalized and tissue-specialized functions. They are considered the core of the CK/PCr system during energy transduction in tissues with high and intermittent energy demands (Sumien et al. 2018; Wallimann et al. 2011) (Table 1).

CK also occurs as macroenzymes (Mifflin et al. 1985; Remaley and Wilding 1989; Sturk and Sanders 1990). Macro-CK type 1 is a complex formed by CK

Table 1 Characteristics of the creatine kinase (CK) isoenzymes

Cellular location	CK isoenzyme	Tissue location
Cytosolic CK	***Homodimer***	
	MM-CK	Skeletal muscle (98%) and heart (70–80%)
	BB-CK	Especially in the brain
	Heterodimer	
	MB-CK	Skeletal muscle (2%), myocardium (20–30%)
Mitochondrial CK	***S-type***	
	Sarcomeric MtCK or sMtCK	Mainly skeletal muscle
	U-type	
	Ubiquitous MtCK or uMtCK	Brain, heart, brown adipose tissue, among several others

isoenzymes (often CK-BB) and immunoglobulin (often monoclonal IgG). Macro-CK type 2 is a polymer of Mt-CK (Lee et al. 1994). These forms of CK are expressed during disease or dysfunction; for example, macro-CK 1 is associated with cardiovascular and autoimmune disease and macro-CK 2 with cancer (Baird et al. 2012).

Concurrently with these discoveries, the CK enzyme and its isoforms had undergone intensive investigation on its potential value in clinical diagnosis since its levels in human serum are altered in several disease states, especially in diseases of the skeletal muscle.

Myopathies are the primary skeletal muscle disorders, and the most important clinically are the dystrophies, polymyositis, myopathies associated with metabolic or endocrine disorders, and drug-induced myopathies. In myopathies, enzymes may be released from the muscle, and increased serum enzyme activity may result (Rosalki 1970). Since the early observation of Sibley and Lehninger (1949) of a high serum aldolase activity in progressive muscular dystrophy, several muscle enzymes, notably lactic dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and CK, were studied and generated crucial clinical information. Ebashi et al. (1959) firstly noted the increased levels of serum CK activity in patients with progressive muscular dystrophies. Many reports since have supported these findings. Okinaka et al. (1959) after measuring the serum CK and aldolase activities in 121 patients with different health conditions, including progressive muscular dystrophy and neuromuscular diseases, liver disease, malignant neoplasms, and acute myocardial infarction, concluded that the determination of the CK activity in the serum might be one of the most valuable tools in the clinical diagnosis of progressive muscular dystrophy given the excellent specificity of CK in this disease. They also suggested the values of CK as an indicator of the development of the disease, being useful in prognosis. Subsequently, studies confirmed the CK activity measurement as a reliable diagnostic tool because of its higher muscle specificity and greater sensitivity (Milhorat et al. 1966; Swaiman and Sandler 1963; Vassella et al. 1965). In a review, Rosalki (1970) described the role of enzyme assays in diseases of the heart and skeletal muscle and pointed to serum CK as the most frequently raised enzyme that shows the most significant degree of elevation in every variety of myopathy, being the choice for the investigation of muscle disease. Bais and Edwards (1982) mentioned other muscle disorders such as Kugelburg-Welander disease, rhabdomyolysis, acromegalic myopathy, hypokalemic periodic paralysis myopathy, viral myositis, and alcohol myopathy, described as causing increased CK activities.

The advances in research utilizing electromyography, nerve conduction studies, and muscle biopsy (Rosalki 1989; Zacks 1970) clarified the role of CK activity in the pathogenesis of muscular diseases, consolidating its importance as a diagnostic tool (Pennington 1980). Increased serum CK level occurred in several other diseases such as cardiac diseases; malignancies; systemic metabolic disorders; thyroid, parathyroid, and hematologic diseases; and alcohol and drug abuse, use of medications like statins and beta-blockers, and physical activity (Lilleng et al. 2011).

There has been extensive discussion in the literature regarding the significance of raised levels of serum CK. The variability in the range of CK considered differences

in the mean levels of activity between males and females, age, physical activity, ethnicity, pregnancy, and various pathological states (Pennington 1980). In addition, it has been shown that the distribution of CK values in a healthy population is markedly skewed toward the higher values and remains non-Gaussian, even after logarithmic transformation of the data (Lev et al. 1999).

Base levels of serum CK in general populations are variable 35–175 U/L with ranges from 20 to 16,000 U/L, and this wide range reflects the inconsistent occurrence of subclinical disorders and minor injury, genetic factors, physical activity status, and medication (Baird et al. 2012). Also, according to the standard established by the International Federation of Clinical Chemistry (IFCC), the upper reference level for CK varies in sex manner, being 171 U/I for males and 145 U/I for females (Sumien et al. 2018). In the absence of specific myocardial or brain infarction, physical trauma, or disease, serum CK levels greater than 5000 U/L are generally considered to indicate serious disturbance to muscle. However, there is no universally agreed or accepted standard (Lilleng et al. 2011).

Creatine Kinase in Statin-Associated Muscle Symptoms

Hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are the most prescribed and effective pharmacological therapy for treating hypercholesterolemia and decreasing the incidence of cardiac events by 20 to 44% for both secondary and primary prevention (Moßhammer et al. 2014; Selva-O'Callaghan et al. 2018; Taylor and Thompson 2018; Thompson et al. 2016). Despite their clinical effectiveness and a good safety profile of statins, their use is associated with a range of skeletal muscle side effects, ranging from mild to severe (Chien et al. 2019; Kromer and Moosmann 2009; Moßhammer et al. 2014; Nguyen et al. 2018).

Statin-associated muscle symptoms (SAMS) are the most reported adverse effect of statins, presented in 7–29% of patients receiving statin therapy in the clinical practice and according to observational studies, respectively (Adhyaru and Jacobson 2018; Chien et al. 2019; Thompson et al. 2016). SAMS contributed significantly to statin nonadherence and discontinuation rates of statin therapy and have been associated with a higher risk of recurrent cardiovascular events (Rallidis 2020). The clinical spectrum of SAMS is highly heterogeneous and ranges from mild weakness, cramps, and muscle pains to very rare and life-threatening rhabdomyolysis. Varying definitions and terms are used to define SAMS and often vary by guidelines (Adhyaru and Jacobson 2018). The American College of Cardiology and the American Heart Association (Pasternak et al. 2002), the Canadian Working Group (Mancini et al. 2013), and the National Lipid Association (Rosenson et al. 2014) have proposed definitions of SAMS based on symptoms and the magnitude of CK elevation (Table 2). The European Atherosclerosis Society has proposed integrating all muscle-related complaints, subdivided by the presence or absence of CK elevation (Table 2) (Stroes et al. 2015).

Understanding variation in CK levels is important for the appropriate administration of statin therapy to all population segments. However, the presence of SAMS

can occur even with normal or slightly elevated levels of serum CK, as observed in Table 2 (Ghatak et al. 2010; Moßhammer et al. 2014; Thompson et al. 2016). Confirmation of SAMS remains a challenge in the absence of validated tests and a specific, sensitive biomarker (Ramachandran and Wierzbicki 2017). Thus, the diagnostic of SAMS is still subjective and based on clinical manifestations (Adhyaru and Jacobson 2018; Chien et al. 2019; Thompson et al. 2016).

Symptoms of statin-related myopathy usually occur soon after initiation of statin therapy. However, symptoms may also appear after years of treatment (Beltowski et al. 2009) and usually dissipate after cessation of therapy, although it may take several months to resolve (Thompson et al. 2016).

Several risk factors can predispose to SAMS, including preexisting risk factors (advanced age, being female, low body mass index, ethnicity, multisystem diseases, alcohol consumption), comorbidities, high doses of statin therapy, and polypharmacy (Adhyaru and Jacobson 2018; Stroes et al. 2015; Thompson et al. 2016). Also, concomitant treatment of statins medication(s) that inhibit cytochrome P450 (CYP450) isoenzymes, organic anion transport protein 1B1 (OATP1B1), or P-glycoprotein 1 (P-gp) has been associated with an increased risk of new or worsening muscle pain (Stroes et al. 2015).

The molecular mechanisms explaining statin myopathy remain unknown, but preexisting deficiencies in energy production might contribute to symptom development. Recent in vivo and in vitro studies have suggested that statin therapy might provoke cellular oxidative stress and impair mitochondrial function and muscular calcium homeostasis, leading to myotoxicity (Mancini et al. 2013). An additional potential mechanism for SAMS development suggested that the inhibition of HMG-CoA reductase also prevents the formation of several intermediate compounds with critical metabolic functions such as prenylated isoprenoids, ubiquinone, or coenzyme Q10 (CoQ10) (Rallidis 2020; Watanabe et al. 2021). As a result, changes in cellular energy metabolism, protein signaling, and mitochondrial function have been proposed (Fig. 2).

Association of Serum Creatine Kinase and Single-Nucleotide Polymorphisms with Statin-Associated Muscle Adverse Events

Considerable interindividual variability in lipid-lowering response to statins is complex and involves numerous factors, including environmental, dietary, and genetic (Romaine et al. 2010). The single-nucleotide polymorphisms (SNPs) or other variations in genes encoding transporters or drug-metabolizing enzymes can significantly impact the distinctive responses to statin use (Romaine et al. 2010).

Several SNPs have been associated with increased incidence of myopathy in patients receiving statins (Table 3 and Fig. 3), ranging from mild myalgia without CK elevation to mild myopathy with mild CK elevation to rhabdomyolysis (Xiang et al. 2018). ABC and SLC are two major families of transporters, where variations in the genes of these transporters have been shown to influence the disposition of statins and the risk of SAMS (Kee et al. 2020).

Table 2 Terminologies for statin-associated muscle symptoms according to expert panels

American College of Cardiology/American Heart Association (Pasternak et al. 2002)
Myopathy: General term referring to any muscle disease *Myalgia*: Muscle ache or weakness without CK elevation *Myositis*: Muscle symptoms with increased CK levels *Rhabdomyolysis*: Muscle symptoms with marked CK elevation (> 10 times the ULN) and with creatinine elevation
Canadian Work Group (Mancini et al. 2013)
Myopathy: General term referring to any muscle disease *Myalgia* Laboratory: CK ≤ ULN Clinical: Muscle ache/weakness *Myositis* Laboratory: CK ≥ ULN Clinical: Muscle ache/weakness *Rhabdomyolysis* Laboratory: CK > 10 times ULN (CK > 10,000 U/L) Clinical: Muscle ache/weakness; renal dysfunction might result from myoglobinuria; need for hydration therapy *HyperCKemia* Laboratory: Mild, Grade 1 CK > ULN, ≤5 times ULN Mild, Grade 2 CK >5 times, ≤10 times ULN Moderate CK >10, ≤50 times ULN Severe CK >50 times ULN Clinical: Mild, Grade 1 Might/might not have myositis Mild, Grade 2 Might/might not have myositis Moderate Might/might not have rhabdomyolysis with/without renal dysfunction Severe Might/might not have rhabdomyolysis with/without renal dysfunction
National Lipid Association (Rosenson et al. 2014)
Myopathy: Muscle weakness (not attributed to pain and not necessarily associated with elevated CK) *Myalgia*: Unexplained muscle discomfort often described as "flu-like" symptoms with normal CK level. The spectrum of myalgia complaints includes: Muscle aches Muscle soreness Muscle stiffness *Myositis*: Muscle inflammation *Myonecrosis/hyperCKemia:* Mild >3-fold greater than baseline untreated CK levels or normative upper limit adjusted for age, race, and sex Moderate ≥10-fold greater than untreated baseline CK levels or normative upper limit adjusted for age, race, and sex Severe ≥50-fold above baseline CK levels or normative upper limit adjusted for age, race, and sex *Rhabdomyolysis*: Myonecrosis with myoglobinuria or acute renal failure (increase in serum creatinine ≥0.5 mg/dL)
European Atherosclerosis Society (Stroes et al. 2015)
Myalgia: Muscle symptoms with normal or mildly elevated CK *Myopathy or myositis*: Muscle symptoms with CK levels (usually) >10x ULN but <40x ULN *Rhabdomyolysis*: Severe muscle symptoms with marked CK elevation (usually >40x ULN) and renal injury

CK creatine kinase, *ULN* upper limit of normal

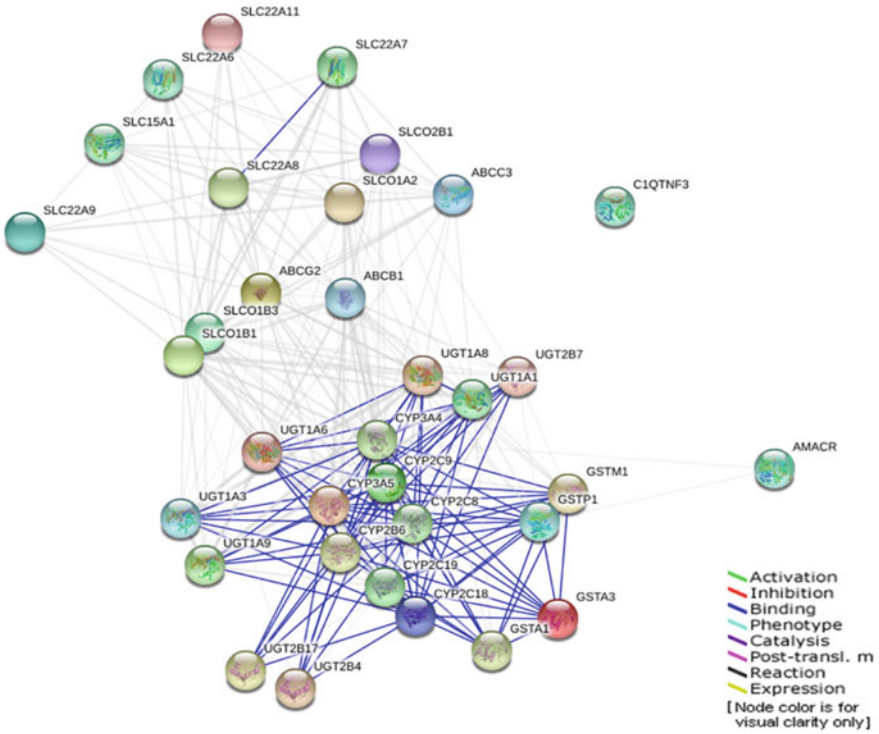

Fig. 2 Representation of the 34 genes involved in the signaling statin pathway. (https://pathcards.genecards.org/Card/statin_pathway_-_generalized_pharmacokinetics?queryString=SLCO1B1)

The primary transporters involved in statin availability are OATP1B1, coded by the solute carrier organic anion transporter family member 1B1 (SLCO1B1) gene (DeGorter et al. 2013), ATP-binding cassette (ABC) transporters subfamily B member 1 (ABCB1/P-gp) coded by the ABCB1 gene (Niemi 2010), and ABC transporters subfamily G member 2 [ABCG2/breast cancer resistance protein (BCRP)] coded by the ABCG2 gene (Björkhem-Bergman et al. 2013).

Considering the critical role of specific intestinal and hepatic transporters in the determination of serum statin concentrations, modification in molecular and genetic levels could influence the metabolization of statins resulting in a higher risk of increased serum CK levels and consequently myopathies (Ferrari et al. 2014; Voora et al. 2009). The gene *SLCO1B1* is a member of the OATPs (previously known as OATP2, OATP-C, liver-specific transporter 1, and SLC21A6) and is the major influx transporter expressed predominantly on the basolateral membrane of human hepatocytes (Niemi 2010). OATP1B1 is the protein coded by the *SLCO1B1* gene located on chromosome 12 and is known to transport several endogenous and exogenous substances, including bile acids, thyroid hormones, methotrexate, and statins (Romaine et al. 2010). A particular genetic polymorphism in *SLCO1B1*, c.521T>C (rs4149056), markedly decreases the function of OATP1B1

Table 3 Polymorphisms in genes participating in the statin signaling pathway, including nomenclature, function, position, gene identification, and substrate/metabolized drug

Gene	Name	Function	GRCh38.p7 Position	ID SNP (rs)	Substrate/drug
SLCO1B1	*Solute carrier organic anion transporter family member 1B1*	Cellular influx of many endogenous and xenobiotic compounds (including the HMG-CoA reductase inhibitors (statins))	chr12: 21131194-21239796	rs11045819	Fluvastatin, rifampin
				rs2291073	Lovastatin
				rs2306283	Atorvastatin, pitavastatin, rifampin
				rs2900478	HMG-CoA reductase inhibitors
				rs4149015	Pravastatin
				rs4149032	Letermovir
				rs4149036	Atorvastatin
				rs4149056	Atorvastatin, lovastatin acid, rosuvastatin, fluvastatin, rosuvastatin, pravastatin, simvastatin, cerivastatin
				rs4149081	Rosuvastatin, simvastatin
				rs4363657	Simvastatin
				SLCO1B1*1, SLCO1B1*5, SLCO1B1*9, SLCO1B1*14, SLCO1B1*15	Pitavastatin, rifampin, rosuvastatin
				SLCO1B1*1A, SLCO1B1*1B	Fluvastatin, atorvastatin, pravastatin
ABCB1	*Superfamily of human adenosine triphosphate (ATP)-binding cassette (ABC) transporters*	Encode transporter and channel proteins that function as efflux pumps	chr7: 87503863-87713323	rs1045642	Atorvastatin
				rs1128503	Simvastatin
				rs2032582	Simvastatin, pravastatin, atorvastatin

(continued)

Table 3 (continued)

Gene	Name	Function	GRCh38.p7 Position	ID SNP (rs)	Substrate/drug
ABCG2	*ABC subfamily G, isoform 2 (ABCG2)*	Encodes the transporter breast cancer resistance protein (BCRP), which is an ATP-binding cassette (ABC) efflux transporter. Plays an important role in drug response	chr4: 88090264-88231417	rs2231142	Simvastatin, atorvastatin, rosuvastatin, fluvastatin
				rs2199939	Rosuvastatin
CETP	*Cholesteryl ester transfer protein*	Involved in the transfer of cholesteryl ester from high-density lipoprotein (HDL) to other lipoproteins	chr16: 56961923-56983844	rs1532624	HMG-CoA reductase inhibitors
				rs4783961	Fluvastatin
				rs5882	Fluvastatin, simvastatin, rosuvastatin
				rs708272	Simvastatin, lovastatin
				rs708272	Atorvastatin, pravastatin, rosuvastatin
COQ2	*4-Hydroxybenzoate polyprenyltransferase*	Encodes an enzyme that functions in the final steps in the biosynthesis of CoQ (ubiquinone), a redox carrier in the mitochondrial respiratory chain, and a lipid-soluble antioxidant	chr4: 83138568-83285129	rs4693075	Atorvastatin, HMG-CoA reductase inhibitors, rosuvastatin
				rs6535454	Atorvastatin, HMG-CoA reductase inhibitors, rosuvastatin
CYP2D6	*Cytochrome P450 2D6*	Involved in the metabolism of up to 25% of the drugs that are in common use in the clinic	chr22: 42125531-42130881	CYP2D6*1, CYP2D6*3, CYP2D6*4	Atorvastatin, rosuvastatin, simvastatin, fluvastatin
				CYP2D6*1, CYP2D6*5, CYP2D6*10	Lovastatin

CYP3A4	*Cytochrome P450 3A4*	Encodes a member of the cytochrome P450 superfamily involved in drug metabolism	chr7: 99756960-99784188	rs4986910	Fluvastatin
				CYP3A4*1, CYP3A4*22	Simvastatin
				CYP3A4*1, CYP3A4*1G	Atorvastatin
				rs35599367	Simvastatin
CYP3A5	*Cytochrome P450 3A5*	It is an important hepatic and extrahepatic pharmacogenetic. Approximately half of the medications that P450 oxidatively metabolizes are CYP3A substrates	chr7: 99648189-99680026	rs776746	Lovastatin, simvastatin
				rs17161788	Atorvastatin

GRCh38.p7 Genome Reference Consortium Human Build 38 patch release 7, *ID* identification, *SNP* single-nucleotide polymorphisms, *rs* SNP reference, *chr* chromosome. Sources: https://www.pharmgkb.org/gene/PA134865839/clinicalAnnotation and https://www.genecards.org

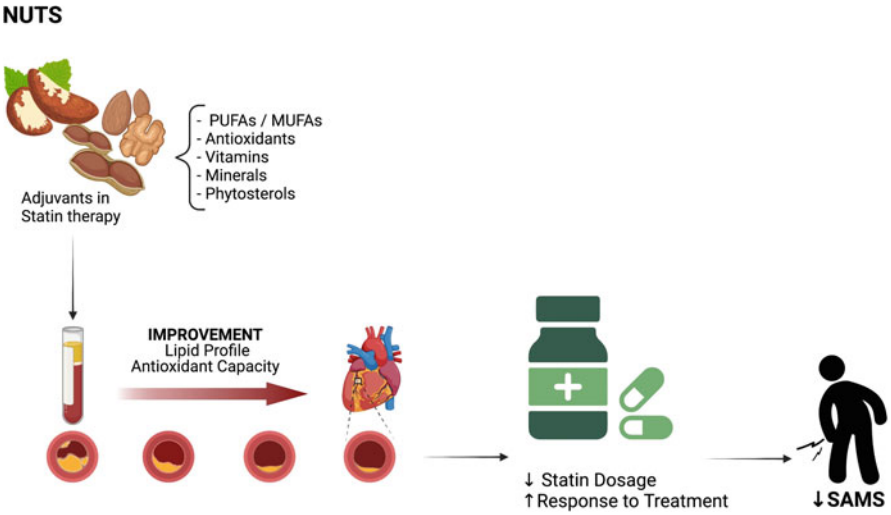

Fig. 3 The potential benefits of nut consumption in statin-associated muscle symptoms (SAMS). Nuts are nutrient-dense foods and in general contain healthy monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) profiles, vitamins, minerals, antioxidants, and phytosterols compounds, with recognized benefits to human health. Considering these benefits, their addition to the diet in a combination of a low-dose or an intermittent dosing statin may allow patients to achieve their lipid goals while limiting toxicity from the drug therapy. (Created with BioRender.com)

(Tirona et al. 2001) in hepatocytes showing a vital role in the cholesterol-lowering effect and SAMS (Xiang et al. 2018), especially linked with the use of simvastatin (Linskey et al. 2020). The T521C SNP reduces the activity of OATP1B1, increasing the statin bioavailability (DeGorter et al. 2013). The presence of at least one allele C (521CC, 521TC, and 521CC+TC from rs4149056 SNP) is associated with increased incidence of SAMS when compared with 521T carriers. Moreover, 521C carriers have improved blood flow into the liver, altered systemic exposure, and higher liver concentrations of statins, changing the lipid-lowering effects and predisposition to muscle toxicity (SEARCH Collaborative Group et al. 2008; Xiang et al. 2018).

The type of statins can also influence the incidence of SAMS in the presence of *SLCO1B1* SNPs. Significant side effects in the presence of *SLCO1B1* SNPs have been associated with altered pharmacokinetics of the simvastatin and an increased risk of myopathy during simvastatin therapy (Xiang et al. 2018).

The rs4363657 SNP is another variant in the *SLCO1B1* gene and was initially detected by the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) with a strong association of myopathy (SEARCH Collaborative Group et al. 2008). This study demonstrated that the polymorphism rs4363657 is in nearly complete linkage disequilibrium with rs4149056, which was previously associated with altered statin pharmacokinetics. The buildup of circulating statin concentration occurs, increasing the susceptibility to muscle toxicity (Seithel et al. 2008). The literature described 17 SNPs identified in the *SLCO1B1*

gene. Of these, the two most studied SLCO1B1 SNPs associated with SAMS are c.388A>G (rs2306283) and c.521T>C (rs4149056). These SNPs occur alone or in combination (Kee et al. 2020; Romaine et al. 2010).

ABCB1 and ABCG2 genes also encode transport proteins that can determine plasma statin concentrations (Ferrari et al. 2014). For the ABCB1 gene, it has been shown that the SNP C3435T (rs1045642), specifically the 3435C genotype, is associated with a higher risk of myalgia (Hoenig et al. 2011; Thompson et al. 2005), while the 1236T genotype is associated with an enhanced efficacy of simvastatin. For the ABCG2 gene, a relatively common SNP is C421A (rs2231142), which has been associated with reduced ABCG2 activity (Bercovich et al. 2006) and increased bioavailability (Keskitalo et al. 2009).

Combinations of SNPs may better predict the occurrence of drug-induced reactions in comparison to individual SNPs (Ferrari et al. 2012). For example, correlations between statin-induced elevations in serum CK level and SNPs in the SLCO1B1, ABCB1, ABCG2, and CYP450 genes, further many other SNPs in transporters, and drug metabolism genes suggest that genotype combination may enable precise identification of patients at risk of statin-induced elevations in serum CK. The C allele in the T521C (rs4149056) SNP of the SLCO1B1 gene and the T allele in the C1236T (rs1128503) SNP of the ABCB1 gene result in the increased bioavailability of atorvastatin, rosuvastatin, and simvastatin (Björkhem-Bergman et al. 2013; DeGorter et al. 2013; Ferrari et al. 2014), elevated serum CK levels (Scarpini et al. 2012), and a higher risk of simvastatin- and rosuvastatin-induced myopathy (Björkhem-Bergman et al. 2013; Ferrari et al. 2014). Contrary to the G allele in the A388G (rs2306283), SNP of the SLCO1B1 gene increases the efficiency of the OATP1B1 transporter, decreasing the bioavailability of atorvastatin, rosuvastatin, and simvastatin (DeGorter et al. 2013) and lowering the risk of statin-induced myopathy (Nies et al. 2013).

Drug-metabolizing enzymes that affect statin pharmacokinetics by impacting oral bioavailability and clearance can alter SAMS risk (Kee et al. 2020). Except for pravastatin, statins are metabolized by the cytochrome P450 pathway, transforming lipophilic compounds into hydrophilic compounds in the liver for excretion (Moßhammer et al. 2014; Thompson et al. 2016). Atorvastatin, lovastatin, and simvastatin are metabolized primarily by cytochrome P450 family 3 subfamily A member 4 (CYP3A4) (Thompson et al. 2016; Adhyaru and Jacobson 2018; Beltowski et al. 2009). Unlike other CYPs, genes from the CYP3A family are not highly polymorphic, accounting for inconsistent findings for association studies of genetic variation for CYP3A4 (Kee et al. 2020). Other statins, such as fluvastatin, pitavastatin, and rosuvastatin, are minimally metabolized by cytochrome P450 family 2 subfamily C member 9 (CYP2C9) and have fewer statin-drug interactions (Adhyaru and Jacobson 2018; Beltowski et al. 2009; Thompson et al. 2016). The CYP2C9 gene has more than 60 reported genetic variants, of which 2 commonly studied CYP2C9 genotypes are the missense variants CYP2C9∗2 (rs1799853) and CYP2C9∗3 (rs1057910) (Kee et al. 2020).

All these genetic variants could be important candidates for the early identification of subjects at risk for the subsequent development of statin-induced CK

elevation. Ferrari et al. (2014) recommended that genotyping at least three SNPs would allow the identification of about 40% of the subjects who will subsequently develop statin-induced elevated serum CK levels. A pharmacogenetic test would result in the improved safety of statin therapy because it can reveal markers that are predictive of statin efficacy or the occurrence of statin-related adverse drug reactions, and it could be helpful in tailoring treatment, based on individual subjects or specific subgroups (de Keyser et al. 2011).

The Potential Health Benefits of Nuts

Innovative nutritional strategies to reduce the main cardiovascular risk factors have been developed, including either dietary changes or consumption of specifically targeted functional foods and dietary supplements to prevent and treat diseases (Hunter and Hegele 2017). Nuts are commonly consumed in the Mediterranean diet, and their consumption has been recommended to populations worldwide (Lima et al. 2019). Due to their nutritional composition, extensive research has been carried out on nuts and health outcomes (Cardoso et al. 2017; de Souza et al. 2017). Tree nuts are defined as dry fruits with one seed in which the ovary wall becomes hard at maturity. Popular tree nuts are Brazil nuts (*Bertholletia excelsa*), almonds (*Prunus amigdalis*), walnuts (*Juglans regia*), pistachios (*Pistacia vera*), cashews (*Anacardium occidentale*), and macadamias (*Macadamia integrifolia*) (Cardoso et al. 2017). They are nutrient-dense foods, each with a unique composition. In general, these foods contain healthy monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) profiles; protein; soluble and insoluble fibers; vitamins E and K; folate; thiamine; minerals such as magnesium, copper, potassium, and selenium; and substances such as carotenoids, antioxidants, and phytosterols compounds, with recognized benefits to human health (de Souza et al. 2017).

The nutrient composition of nuts is a pivotal contributor to low-density lipoprotein cholesterol (LDL-C)-lowering and high-density lipoprotein cholesterol (HDL-C)-preserving effects (Coates et al. 2018). Antioxidant compounds in nuts have been shown to limit oxidative damage. Specifically, polyphenols in nuts have antioxidant properties, which can modulate nitric oxide (NO) production, thereby altering vascular function. Also, oxidative stress may be controlled by nonenzymatic nutrients such as vitamins A, C, and E and minerals copper, zinc, manganese, and selenium (Barbosa et al. 2010). These nutrients could act as cofactors for antioxidant enzymes or directly as a protective factor against oxidative stress.

Studies incorporating nut consumption and the side effects of statin use, especially considering SAMS, are scarce in the literature. Generally, expert panels recommend nut consumption as part of guidelines for lifestyle changes, targeting the prevention of cardiovascular diseases (Arnett et al. 2019).

Given its antioxidant, anti-inflammatory, chemopreventive, and antiviral potential, selenium has been extensively studied about disease prevention and treatment (Wrobel et al. 2016; Zoidis et al. 2018). The health-related properties of selenium are due to its unique incorporation mechanism into selenoproteins and include

protection against cancer, proper thyroid function, and protection against cardiovascular and muscle disorders (Zoidis et al. 2018). Among all edible nuts, Brazil nuts have the highest selenium concentration. A single unit can have up to 400 μg of selenium, considering one of the most concentrated selenium food sources (Cardoso et al. 2017). Also, the main chemical form of selenium in Brazil nuts is selenomethionine, which has a high bioavailability and low toxicity (Donadio et al. 2019; Lima et al. 2019; Weekley and Harris 2013). The potential health benefits of Brazil nut consumption included but were not limited to antioxidant and anti-inflammatory effects, reduction of cardiovascular disease risk factors, anticancer effects, improvement of cognitive performance in older adults (Rita Cardoso et al. 2016), prevention of oxidative DNA damage (Cominetti et al. 2011; Macan et al. 2020), and potential prebiotic properties (Sugizaki and Naves 2018). Also, the benefits of Brazil nut consumption could be extended to SAMS since it contributed to the improvement of oxidative stress parameters, which could be associated with decreased serum CK activity, improving the muscle homeostasis of patients using statins (Watanabe et al. 2020).

Dietary therapy has an impressive impact on cardiovascular events and can be helpful as adjunctive therapy to medication (Bruckert and Rosenbaum 2011; Sorrentino 2012). Considering the health benefits of nuts, their addition to the diet in a combination of a low-dose or an intermittent dosing statin may allow patients to achieve their lipid goals while limiting toxicity from the drug therapy (Sorrentino 2012). In fact, for each weekly serving of nuts, there is an 8.3% reduction in the risk of death from cardiovascular diseases, demonstrating their cardioprotective potential to use in the global strategy of patients' treatment (Bruckert and Rosenbaum 2011).

Conclusion

CK levels, SAMS, and the consumption of nuts at the first moment are complex topics to be connected. However, when considering that (a) the assessment of CK levels has been used for several years as a biomarker of muscle disorders; (b) in the diagnosis of SAMS, serum CK levels are commonly used; and (c) the consumption of nuts can influence the levels of oxidative stress, altering the homeostasis of CK in the context of SAMS, all concepts interconnect and start to interact in a meaningful way. In this sense, all information presented here brings evidence about these topics, demonstrating their importance and how their interconnection can impact the health/disease binomial.

Applications to Other Diseases or Conditions

This book chapter reviews the creatine kinase (CK) as a biomarker for muscle disorders, especially statin-associated muscle symptoms (SAMS). However, we can extend the applicability of CK as a biomarker to other health conditions, such as in the prediction of severity of the coronavirus disease 2019 (COVID-19).

The COVID-19 pandemic has been a scientific, medical, and social challenge (Ponti et al. 2020). The complexity of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) imposed an urgent need for reliable biomarkers related to COVID-19 disease progression (Ponti et al. 2020). A pattern of hematologic, biochemical, inflammatory, and immune biomarker abnormalities has been identified in patients with more severe degrees of the disease (Ponti et al. 2020). Besides the respiratory symptoms, several neurological and neuromuscular symptoms have been identified as part of the COVID-19 spectrum, including muscle pain and fatigue (Orsucci et al. 2021). Accordingly, studies have noticed that muscular markers were elevated in patients with both severe and fatal COVID-19, and increased CK could be associated with a more severe prognosis in COVID-19, although the precise mechanisms are still unknown.

Mini-Dictionary of Terms

- **Statin**: A class of lipid-lowering medications reduces illness and mortality in those at high risk of cardiovascular disease.
- **Statin-associated muscle symptoms**: Generally defined as all muscle-related complaints such as myalgia, cramps, and perceived weakness.
- **Genetic variability**: The tendency of individual genetic characteristics in a population to vary from one another.
- **Single-nucleotide polymorphism**: A germline substitution of a *single nucleotide* at a specific position in the genome.
- **Nut**: A fruit consisting of a hard nutshell protecting a kernel which is usually edible.

Key Facts About Creatine Kinase

Creatine kinase is an enzyme widely distributed in tissues.

Creatine kinase (CK) exists under various isoenzymes localized differentially on a subcellular level, and these specific locations are essential for the functioning of the CK network.

Creatine kinase is a central controller of cellular energy homeostasis.

Creatine kinase is expressed at high levels in cells with high energy requirements.

Creatine kinase and its isoenzymes in serum are widely used for diagnostic purposes in muscular disorders.

Summary Points

- Creatine kinase has been widely used as a biomarker in clinical practice to identify muscle disorders and other health conditions.

- Studies about its structures and functions have advanced over time and provided important tools for clinical practice.
- One of the most expressive uses of creatine kinase is in identifying and treating statin-associated muscle symptoms.
- The single-nucleotide polymorphisms in genes related to statin metabolism pathways can be highlighted among the risk factors predisposing to treating statin-associated muscle symptoms.
- Nut consumption has been widely associated with health benefits as promising adjuvants to drug therapies, improving health parameters such as lipid profile and oxidative stress, and decreasing serum creatine kinase levels.

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Circulating Glutamate as a Potential Biomarker of Central Fat Accumulation and Concomitant Cardiometabolic Alterations

46

Ina Maltais-Payette and André Tchernof

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Abstract

Accumulation of excess lipids in ectopic locations such as visceral adipose tissue (VAT), liver, skeletal muscle, and pancreas contributes to a higher risk of cardiometabolic diseases or complications including steatohepatitis, type 2 diabetes, and heart diseases. In the last decade, many studies have reported that circulating concentration of the amino acid glutamate is positively associated with measurements of the amount of visceral or abdominal fat. Therefore, this analyte has been suggested as a potential biomarker for these conditions. Yet, the pathophysiology of the association between circulating glutamate and visceral/abdominal obesity has not been elucidated. Dietary glutamate is extensively used by enterocytes, resulting in a very small proportion reaching the bloodstream. Therefore, increased dietary glutamate intake, in the form of monosodium glutamate or other, is unlikely to explain the association between circulating glutamate and central adiposity. Branched-chain amino acids are known to be elevated in obesity through down-regulation of their catabolism in this condition. Because glutamate is a by-product of this pathway, and not a substrate, it is unlikely that lower BCAA catabolism increases circulating glutamate levels. Additional pathways may contribute to explain the link between glutamate and abdominal, visceral obesity. The uncertainty regarding the basis of this association underscores the necessity of performing additional research to firmly establish the biological plausibility and biomarker potential of circulating glutamate concentration.

Keywords

Glutamate · Abdominal obesity · Type 2 diabetes · Cardiovascular diseases · Liver steatosis · Branched-chain amino acids · MSG

Abbreviations

aKG	Alpha-ketoglutarate
BCAAs	Branched-chain amino acids
BCAT	Branched-chain aminotransferase
BCKA	Branched-chain ketoacid
BCKD	Branched chain ketoacid dehydrogenase
BMI	Body mass index
CT	Computed tomography
CVD	Cardiovascular diseases
DXA	Dual energy absorptiometry
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
LDL	Low-density lipoprotein
MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
MSG	Monosodium glutamate
NAFLD	Nonalcoholic fatty liver diseases

SAT	Subcutaneous adipose tissue
SD	Standard deviation
T2D	Type 2 diabetes
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
WC	Waist circumference

Introduction

Obesity and Body Fat Distribution

According to most recent Canadian guidelines, obesity is defined as a "complex chronic disease in which abnormal or excess body fat (adiposity) impairs health, increases the risk of long-term medical complications and reduces lifespan" (Wharton et al. 2020). The most common metric to assess obesity is the body mass index (BMI), calculated as body weight divided by squared height. According to standard thresholds, individuals with a BMI greater than 30 kg/m^2 are categorized as obese (WHO 2018). The prevalence of obesity has doubled worldwide between 1980 and 2015 to reach 603.7 million people worldwide (Afshin et al. 2017). This is concerning since individuals living with obesity have a higher incidence of type 2 diabetes (T2D), cardiovascular diseases (CVD, namely, hypertension, coronary artery disease, stroke), and many cancers (Guh et al. 2009) as well as reduced quality of life (Therrien et al. 2011) compared to those who are lean.

Even if obesity is correlated with increased health risks, this association is very heterogeneous: some people with obesity may be characterized by significant health problems related to their excess body weight, whereas others appear to be relatively protected, at least in the short term (Neeland et al. 2019). One major factor underlying such heterogeneity is regional body fat distribution.

In the context of a positive energy imbalance, triglycerides are synthesized in adipose compartments, preferentially the subcutaneous adipose tissue (SAT) depots (see Fig. 1). Yet, the expandability of SAT is highly variable (Taylor and Holman 2015). High storage capacity in SAT is more frequent in women and usually generates a gynoid body shape. This pattern often relates to a normal cardiometabolic risk profile, even in the context of high overall adiposity levels (Tchernof and Despres 2013). On the other hand, limited SAT expansion capacity is more frequent in men and generates an android body shape. This pattern is due to increased lipid storage in visceral adipose tissue (VAT) depots as well as in ectopic locations such as skeletal muscle, the liver, the pancreas, the kidneys, and the heart (Fig. 1).

Metabolic Impact of Visceral Obesity

The android body fat distribution pattern is often observed in concomitance with a number of cardiometabolic alterations and these associations are independent of the degree of overall adiposity. The metabolic profile of individuals from both sexes who

show elevated VAT accumulation describes an inability to manage excess diet-derived energy substrates (glucose, fatty acids and amino acids). This is reflected by resistance to insulin action and poor glucose disposal, accompanied by compensatory insulin secretion and/or hyperglycemia, and increased risk for type 2 diabetes (Tchernof and Despres 2013; Neeland et al. 2019). Altered markers of glucose homeostasis include increased fasting glucose and insulin levels, a high homeostatic model assessment of insulin resistance (HOMA-IR) index (Matthews et al. 1985), and elevated percent glycated hemoglobin (HbA1c). In terms of fatty acid metabolism, the limited ability of adipose tissue compartments to expand is reflected by elevated levels of plasma triglycerides, even in the fasting state, which also relates to decreased high-density lipoprotein (HDL)-cholesterol levels. This dyslipidemic state increases the triglyceride content of all plasma lipoproteins: chylomicrons as well as very-low, low-, and high-density lipoproteins (VLDL, LDL, and HDL) (Despres and Lemieux 2006). Limited fatty acid uptake in adipose tissues in the postprandial state also creates a spillover of those fatty acids toward normally lean tissues, as mentioned earlier (Fig. 1) (Carpentier 2008).

Consistent with the deleterious impact of visceral obesity on metabolism, VAT accumulation is more strongly associated with the risk of T2D and CVD than SAT

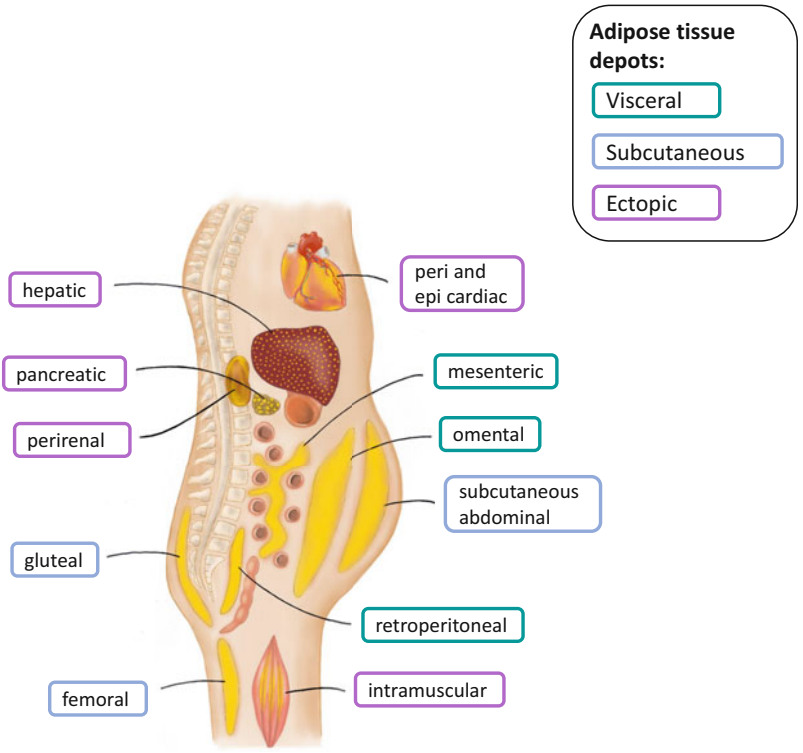

Fig. 1 Localization of the main adipose tissue depots and ectopic lipid storage sites

accumulation (Balkau et al. 2007). During weight loss intervention, the loss of VAT is a better predictor of cardiometabolic improvements than the loss of SAT (Borel et al. 2012). Fortunately, visceral fat has been shown to be mobilized preferentially compared to other compartments during weight loss (Borel et al. 2012).

Measurement of Body Fat Distribution

The gold standard for body fat distribution measurement are imagery methods such as computed tomography (CT) or magnetic resonance imaging (MRI) (Shuster et al. 2012). These are the only approaches allowing differentiation of abdominal subcutaneous and visceral fat. However, they are expensive, time consuming, and in the case of CT, involve radiation. Dual-Energy Absorptiometry (DXA) allows trunk fat quantification without differentiating SAT and VAT specifically (Shuster et al. 2012), but provides calculated VAT estimates in some studies. In a clinical context, the most frequently used tool to assess preferential abdominal fat accumulation is the measurement of the waist circumference (WC) (Neeland et al. 2019).

Amino Acids, Obesity, and Cardiometabolic Diseases

The study of circulating amino acid levels in obesity gained momentum in 2009 with an article by Newgard and collaborators (Newgard et al. 2009). In this study, they compared the metabolomic profile of lean and obese individuals. Using principal component analysis, they found that the factor most different between the two groups was composed of the three branched-chain amino acids (BCAAs, namely, leucine, isoleucine, and valine), methionine, glutamate/glutamine ratio, phenylalanine, tyrosine, and 2 acylcarnitines (C3 and C5). Moreover, this factor was strongly and positively associated with the HOMA-IR index. In the last decade, BCAAs have been extensively studied and have been confirmed to be associated with obesity and insulin resistance as well as T2D and CVD risk (White and Newgard 2019).

In 2017 Okekunle et al. performed a review of the literature on amino acid alterations associated with obesity, T2D, and the metabolic syndrome (MetS) (Okekunle et al. 2017). As can be seen in their summary presented in Fig. 2, most amino acids are affected by 1 or more of these conditions. Amino acids affected by all three include valine, leucine, glutamate, and proline (higher in disease state), as well as glycine (lower in disease state).

In a recent review, Trico et al. reported that patients with nonalcoholic fatty liver disease (NAFLD) had higher levels of BCAAs, alanine, glutamate, lysine, phenylalanine, proline, tryptophan, and tyrosine as well as lower levels of glycine and serine compared to controls (Tricò et al. 2021).

In terms of weight loss, Tulipani et al. reviewed the metabolomic changes induced by weight loss, either with bariatric surgery or behavioral changes (Tulipani et al. 2016). Metabolites lowered by both interventions include phenylalanine, tyrosine, isoleucine, leucine, alanine, and ornithine.

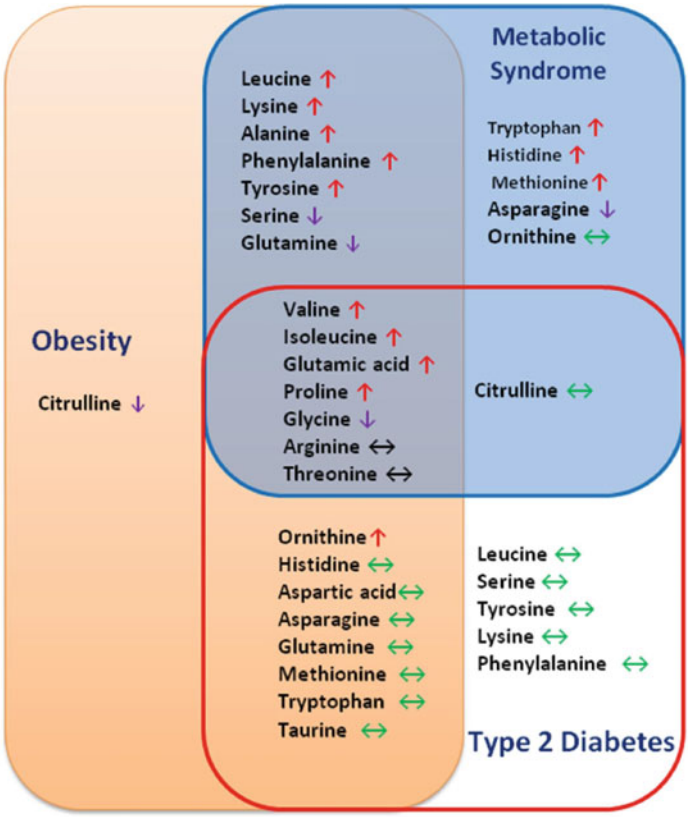

Fig. 2 Summary of a meta-analysis on differences in circulating amino acids between individuals with and without obesity, the metabolic syndrome, and type 2 diabetes. This figure was published in Diabetes Res Clin Pract, Vol 132, Okekunle et al. "Abnormal circulating amino acid profiles in multiple metabolic disorders" pp. 45–58, Copyright Elsevier (2017)

Obesity and the commonly associated cardiometabolic alterations are correlated with changes in circulating amino acids. However, whether these alterations in different diseases are all related to a common factor, for example, overall adiposity or ectopic fat accumulation, is not yet clear. Moreover, the usefulness of amino acids as biomarkers, compared to, or in addition to, traditional screening tools, has yet to be proven. Finally, the pathophysiology and the direction of causality of the association between plasma amino acids and obesity/cardiometabolic alterations needs more investigation.

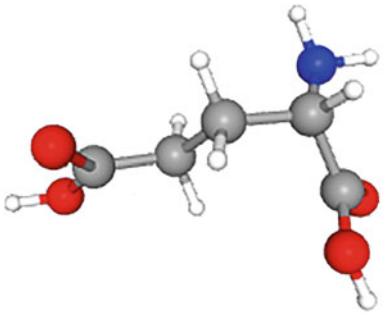

Fig. 3 The 3D structure of glutamate. Publicly available from pubchem.ncbi.nml.nih.gov.

What Is Glutamate?

Glutamate is a polar amino (Fig. 3). It can be synthesized from α-ketoglutarate, through the addition of an amino group by *glutamate dehydrogenase* or an amino-transferase enzyme (Brosnan and Brosnan 2013). It can also be synthesized from other amino acids such as glutamine, arginine, ornithine, proline, and histidine (Brosnan and Brosnan 2013).

Like all other amino acids, glutamate can be used as a building block for protein synthesis, but it is also implicated in a large number of metabolic pathways (Brosnan and Brosnan 2013). In the brain, it is a neurotransmitter and a substrate for neurotransmitter synthesis; in the liver, it is implicated in the urea cycle and in glutathione synthesis; in pancreatic β-cells it stimulates insulin secretion. Glutamate is also implicated in reactions occurring throughout the body such as amino acid transamination and anaplerosis.

Glutamate and Central Adiposity

As mentioned, circulating glutamate concentration is significantly higher in people with obesity than in those who are lean (Okekunle et al. 2017). However, one of the particularities of this analyte is that its levels are more strongly associated with central fat accumulation than with overall adiposity.

Kimberly et al. investigated the metabolomic profile of NAFLD in 997 participants of the Framingham Heart Study Generation 3 (Kimberly et al. 2017). Their first analysis tested the association between metabolites and various cardiometabolic traits. In age- and sex-adjusted analyses, the second metabolite most strongly associated with WC was glutamate (the first being uric acid).

Takashina et al. studied 83 Japanese subjects with normal glucose tolerance to determine the association between plasma amino acids and body fat distribution (Takashina et al. 2016). The amount of VAT and SAT was measured by MRI. Among

all amino acids, glutamate was the most strongly associated with BMI, WC, and VAT. Interestingly, it was not significantly correlated with SAT.

Yamakado et al. investigated whether VAT accumulation was associated with an altered amino acid profile in 1449 Japanese men (Yamakado et al. 2012). VAT and SAT areas were measured by computed tomography (CT) scans. Once again, the amino acid most strongly associated with VAT was glutamate. In that study, glutamate was also associated with SAT, but to a lesser extent. The authors identified men presenting visceral obesity as having a CT-measured VAT area > 100 cm^2 and calculated the ability of each amino acid to differentiate patients with and without visceral obesity. They reported that glutamate was the best amino acid in this regard, with a precision of 75%.

Zhao et al. investigated the metabolic profile of American Indians in the Strong Heart Family Study ($n = 431$) (Zhao et al. 2016). They identified seven metabolites significantly associated with WC after adjustment for age, sex, site, lifestyle socio-economic status, diet, and HOMA-IR. The strongest predictor was a composite of glycine, valine, arginine, and glycine; the second strongest was glutamate.

Menni et al. investigated the role of central fat in cardiometabolic health using metabolomics in 2401 women from the TwinsUK cohort (Menni et al. 2016). They found glutamate to be the strongest independent predictor of DXA-measured trunk fat in a backward linear regression analysis.

Boulet et al. studied a sample of 59 healthy middle-aged women to investigate the metabolomic profile associated with visceral obesity (Boulet et al. 2015). Out of almost 200 metabolites measured, glutamate was the most strongly associated with VAT area after adjustment for total body fat mass. Moreover, it was significantly associated with the mean size of adipocytes from the visceral, but not the subcutaneous compartment. In a secondary analysis of this sample, Maltais-Payette et al. investigated the potential of glutamate as a biomarker for visceral obesity (Maltais-Payette et al. 2018). Glutamate had an accuracy of 78% to distinguish women with and without visceral obesity.

In a second study, Maltais-Payette et al. investigated glutamate's ability to differentiate participants with and without abdominal obesity, defined with a WC measurement greater than 95 cm (Maltais-Payette et al. 2019). The accuracy of glutamate to distinguish these groups was 90% in both men and women.

In the TwinsUK cohort, Maltais-Payette et al. identified participants with abdominal obesity using trunk fat measurements by DXA (Maltais-Payette, unpublished). Individuals with more than 37.7% of fat in the trunk region were considered has having abdominal obesity. Individuals in the fifth circulating glutamate quintile had a seven-fold higher risk of presenting abdominal obesity than those in the first quintile.

There is substantial evidence that circulating glutamate is associated with central fat accumulation. Interestingly, glutamate appears to be much less associated with SAT and overall adiposity. However, it needs to be highlighted that glutamate is measured less frequently than other amino acids. Therefore, some studies investigating plasma amino acids and body fat distribution do not report results on glutamate.

Glutamate and Weight Loss

The impact of weight loss on circulating glutamate levels as been investigated in a few studies, but the results are ambiguous. For example, Aasheim et al. reported that glutamate decreased significantly and linearly 2, 6, and 12 months after bariatric surgery (Aasheim et al. 2011). Liu et al. also reported a significant glutamate decrease 3 months after gastric bypass (Liu et al. 2017). However, Nicolleti et al. saw an increase in glutamate 3 months after surgery, which returned to baseline after 6 months (Nicoletti et al. 2013).

Dietary interventions have also been reported to cause changes in circulating glutamate. Geidenstam reported a lower glutamate level in participants after 3 months of dietary-induced weight loss compared to baseline and no change in glutamate level during a 6-month weight maintenance period (Geidenstam et al. 2017). Zeng et al. also reported that glutamate level was significantly lower in participants after they lost weight in the POUNDS LOST trial (n = 774) and the DIRECT trial (n = 318) (Zheng et al. 2016a). However, Tochikubo reported that circulating glutamate was not significantly changed by a 3-month diet and exercise intervention (Tochikubo et al. 2016).

Unfortunately, none of these studies have investigated the loss of central fat specifically in relation to changes in circulating amino acids. This would be particularly interesting for glutamate, because it is associated with central fat more strongly than with overall adiposity.

Glutamate and Cardiometabolic Diseases

Insulin Resistance and T2D

Labonte et al. investigated the association between fasting plasma amino acids and insulin resistance (Labonte et al. 2017). The latter was measured by hyperinsylinemic-euglycemic clamp, where circulating insulin is artificially progressively increased and glucose is infused to maintain euglycemia. The amount of glucose infused is a gold-standard measurement of insulin sensitivity. In total, 134 participants were studied: 51 lean and healthy, 31 overweight/obese and healthy as well as 52 overweight/obese with T2D. Glutamate, as well as the 3 BCAAs and lysine, were all higher in the overweight/obese healthy group than in the lean-healthy group, and even higher in the overweight/obese T2D group. Seven amino acids were inversely associated with insulin sensitivity and glutamate showed the strongest correlation.

As mentioned above, in the meta-analysis by Okekunle et al., the results from six studies indicated that circulating glutamate level was significantly higher in individuals with vs those without T2D (Okekunle et al. 2017).

In a retrospective analysis of 601 participants from the Framingham Heart Study free of T2D at baseline, Cheng et al. investigated the association between

glutamate as well as glutamine and T2D incidence at 12 years. They reported that baseline glutamate was associated with a higher incidence of T2D. However, this finding was not reproduced in the Malmo Diet and Cancer study cohort. A similar study was done in the PREDIMED trial (Liu et al. 2019), a study designed to evaluate the impact of a Mediterranean diet on participants at high CVD risk. In total, 892 participants were included in these analyses, all non-diabetic at baseline. Glutamate was measured at baseline and 1 year, and T2D incidence after 4.8 years median follow-up was compared between amino acid quintiles. Individuals with the highest baseline glutamate level had six times higher risk of T2D incidence at follow-up compared to individuals with the lowest level. This analysis was adjusted for age, sex, BMI, smoking, physical activity, dyslipidemia, hypertension, baseline BCAA levels, and baseline fasting glucose. The circulating glutamate changes between baseline and 1 year were not predictors of T2D incidence.

NAFLD

One of the first study exploring circulating amino acids in the context of NAFLD was done by Kalhan and collaborators in 2011 (Kalhan et al. 2011). They included 11 patients with simple steatosis, 24 with steatohepatitis, and 25 healthy controls matched for age and sex with the NAFLD participants. Amino acids higher in steatosis versus controls were glutamate, lysine, tyrosine, and isoleucine. Those higher in steatohepatitis than in controls were glutamate, aspartate, tyrosine, phenylalanine, and the three BCAAS. Only five metabolites were significantly different between steatosis and NASH: undecanoate, linolenate, glutamate, creatine, and pyruvate. A random forest analysis was done to discriminate NAFLD patients from control. The topmost contributing metabolite in the algorithm was glutamate, followed by two metabolites implicated in glutathione metabolism (gamma-glutamyltyrosine and cystein-glutathione-disulfite).

A similar study by Gaggini and collaborators (Gaggini et al. 2018) enrolled 44 patients with NAFLD, of which 15 were obese and 29 nonobese, as well as 20 controls without hepatic disease. Although their BMIs were similar, nonobese NAFLD individuals had significantly higher circulating alanine, valine, isoleucine, and glutamate than controls. These differences were also observed between obese-NAFLD and controls, in addition to higher leucine, tyrosine, phenylalanine, and lysine as well as lower glycine.

Qi and collaborators focused on blood metabolites able to differentiate NASH and simple steatosis (Qi et al. 2017). They recruited 38 patients with steatosis, 21 with NASH and 31 controls. The mean BMI of the three groups were very similar and most patients were severely obese, with the average BMI over 45 kg/m^2 for all groups. The authors performed untargeted metabolomics and identified the metabolites most different between simple steatosis and NASH patients. The top 5 were pyroglutamate, glutamine, glutamate, uracil, and α-linoleic acid.

Cardiovascular Diseases

The association between plasma glutamate and glutamine and cardiovascular disease incidence was studied in the PREDIMED trial by Zheng et al. (2016b). In the fully adjusted model (age, sex, family history of CVD, smoking, BMI, hypertension, dyslipidemia, and T2D), 1 standard deviation (SD) increase in circulating glutamate was associated with a 43% increased risk of CVD at follow-up (median 4.8 years).

Kume et al. investigated the predictive ability of circulating amino acids for CVD in Japanese diabetic patients (Kume et al. 2014). They followed patients for CVD up to 10 years. Circulating glutamate was not significantly different between cases and controls. However, using a machine learning algorithm, the authors constructed an optimal CVD-predicting amino-acid index, and glutamate was one of the six metabolites in this index. This index was significantly higher in cases vs controls, and its ability to distinguish cases from controls was 72%. One SD increment of this index was associated with a 286% higher risk of CVD (model adjusted for age, systolic blood pressure, hypertension, HDL-cholesterol, kidney function, and arterial stiffness).

Vaarhorst et al. compared the CVD-predicting ability of a metabolite-based score to traditional risk factors (Vaarhorst et al. 2014). The metabolite score included glutamate, among 12 other molecules. The traditional risk factors were age, total cholesterol, HDL-C, systolic blood pressure, BMI, sex, smoking, T2D, and parental history of myocardial infarction. One SD increment of the metabolite-score was associated with a 91% higher risk of CVD. After adjustment for the traditional risk factors, the score was still significantly associated with CVD incidence, with a 50% increased risk per SD. The ability to distinguish cases from controls was 75% for the metabolite score vs 82% for the traditional risk factors. Interestingly, when comparing the risk of CVD associated with each of the 36 metabolites measured, glutamate had the second-best predicting ability after lipids.

Partial Summary

Plasma glutamate is altered in states of insulin resistance, T2D, and NAFLD. It is also a predictor of long-term incidence of T2D and CVDs. The lipid spillover caused by the saturation of SAT is responsible for fat accumulation in VAT, the liver, the pancreas, skeletal muscle, and the blood vessels. This lipid accumulation directly and indirectly participates to the development of T2D, NAFLD and CVDs (Tchernof and Despres 2013). Moreover, each of these diseases increases the risk for the others. Therefore, it is difficult to identify why glutamate is elevated in each of these conditions.

Pathophysiology

The pathophysiology of the association between circulating glutamate and abdominal obesity/cardiometabolic diseases has never been investigated specifically. However, there are results from study subjects related to glutamate or other amino acids

that provide information on potential mechanisms. It also allows identification of some mechanisms that are not likely involved.

Genetics

Maltais-Payette et al. investigated a subgroup of identical twins from the TwinsUK cohort that were discordant for trunk fat percentage [Maltais-Payette unpublished]. When circulating glutamate was compared between leaner and heavier twins of such pairs, heavier twins were found to have higher circulating glutamate. This indicates that for a given genetic background, having more trunk fat is associated with higher plasma glutamate.

In 2014, Shin et al. published a summary of the heritability of hundreds of metabolites (Shin et al. 2014b). To do so, they computed the similarity in each metabolite between the individuals of twin pairs. Then, they compared the similarity between identical and fraternal twin pairs. It is assumed that the difference in similarity is due to the fact that identical twins share almost 100% of their genetics, whereas fraternal twins share only 50%. In these calculations, glutamate was found to be 25% heritable, which is lower than most of the other amino acids. For example, the heritability of leucine, isoleucine, and valine was calculated to be 43%, 49%, and 41%, respectively.

Dietary Glutamate and MSG

Glutamate is a nonessential amino acid, meaning that it is synthesized endogenously and does not need to come from the diet (Brosnan 2000). Nevertheless, glutamate is present in many food items, both as part of proteins and in its free form. Free glutamate is naturally present in food and can be added through monosodium glutamate (MSG), a flavor additive (Brosnan 2000).

It has been suggested that MSG could cause weight gain and obesity. Part of this notion stems from epidemiological studies reporting that individuals with the highest MSG consumption had greater prevalence of overweight or obesity compared to those with the lowest consumption (He et al. 2011). However, the high MSG-consumers also had higher dietary intakes, lower physical activity scores, and higher incomes than the low MSG-consumers. Moreover, another epidemiological study reported contradictory results: individuals with the greatest MSG intakes had lower BMIs than those with the lowest MSG intakes (Shi et al. 2010). Overall, the epidemiological link between MSG intake and obesity is probably due in large part to the nutritional transition occurring in many Asian countries since MSG is mostly added to processed food (Brosnan et al. 2014).

Early experiments showed that repeatedly injecting very large doses (3000 mg/kg/day) of glutamate in newborn mice caused them to grow short and obese (Olney 1969). The dosage injected to mice in this study was 15 to 20 times higher than usual dietary glutamate intake (Brosnan et al. 2014). Moreover, humans

consume glutamate in their diet and not through injections. Experiments showed that only a very small fraction (~5%) of dietary glutamate reaches the blood stream because most of it is oxidized in enterocytes (Reeds et al. 2000). Concordantly, even high dietary glutamate intakes do not raise circulating glutamate (Tsai and Huang 1999).

Overall, dietary glutamate is presumably not responsible for the positive association between glutamate and central fat accumulation.

Microbiota

The gut microbiota has also been suggested to be implicated in the relationship between circulating glutamate and adiposity. This hypothesis came from the observation that the abundance of a specific gut bacteria species, *Bacterioides thetaiotamicrion*, was inversely correlated with plasma glutamate concentration (Liu et al. 2017). To test for a causal mechanism, investigators gavaged this bacteria to mice combined with a diet rich in fat and sugar to induce obesity. They reported that mice receiving the bacteria had a lesser weight gain and lower circulating glutamate than those receiving placebo. Moreover, the abundance of this bacteria species was lower in individuals who were obese compared to those who were lean, and it increased following weight loss induced by bariatric surgery. Finally, this increase in *Bacterioides thetaiotamicrion* was correlated with a decrease in circulating glutamate level (Fig. 4).

The proposed mechanism is that people living with obesity have lower amounts of a bacteria species catabolizing glutamate, causing glutamate accumulation in the intestine. As mentioned earlier, glutamate in the gut does not reach the circulation,

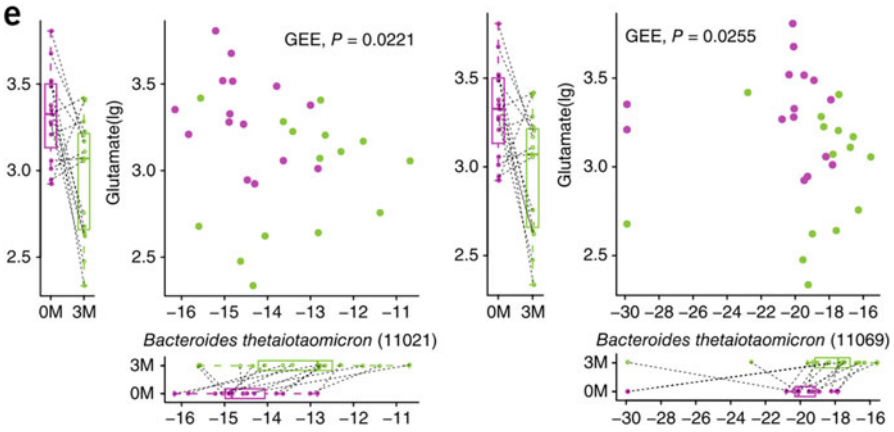

Fig. 4 Microbial and metabolic alterations during weight-loss intervention by sleeve gastrectomy. (This figure was published in Nat Med, Vol 23, Liu et al. "Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention", pp. 859–868, Copyright Springer Nature (2017))

therefore more investigation is warranted to determine whether microbiota changes could modulate circulating glutamate and how.

BCAA Catabolism

As mentioned in the introduction, BCAAs are elevated in obesity (Newgard 2017). Multiple studies have demonstrated that a downregulation of their catabolism is contributing to this phenomenon (Newgard 2017). Because glutamate is a by-product of this pathway, it has been suggested that its elevation in abdominal obesity could also be a consequence of the altered BCAA catabolism.

The first two steps of BCAA catabolism are common to all three amino acids (Fig. 5). First, the *branched-chain aminotransferase* (BCAT) transfers an amino group from the BCAA to an alpha-ketoglutarate (aKG). This reaction produces a branched-chain ketoacid (BCKA) and a molecule of glutamate. Second, the BCKA is decarboxylated by the *branched-chain ketoacid dehydrogenase* (BCKD) complex into an acyl-CoA. An important characteristic of BCAA catabolism is that this process is highly compartmentalized (Biswas et al. 2019). Indeed, BCAT is mostly expressed in peripheral tissues such as skeletal muscle and adipose tissue, so that dietary BCAAs largely bypass the liver first-pass. The BCKA produced in peripheral tissues returns to the circulation and to the liver, where BCKD is highly expressed, to continue their catabolism (Fig. 5).

Regarding adipose tissue, She et al. showed that murine models of obesity (both Zucker fatty rats and ob/ob mice) had significantly lower BCAT protein abundance and enzymatic activity than lean controls (She et al. 2007). Moreover, in human participants, they observed that BCAT protein abundance increased after bariatric surgery and that this was accompanied by a decrease in circulating BCAA levels. Various studies have now confirmed that BCAT is less abundant, expressed, and active in adipose tissue in the context of obesity (Biswas et al. 2019). Results on BCKD are more ambiguous, but since it has low expression in this organ, the relevance of potential changes is not clear.

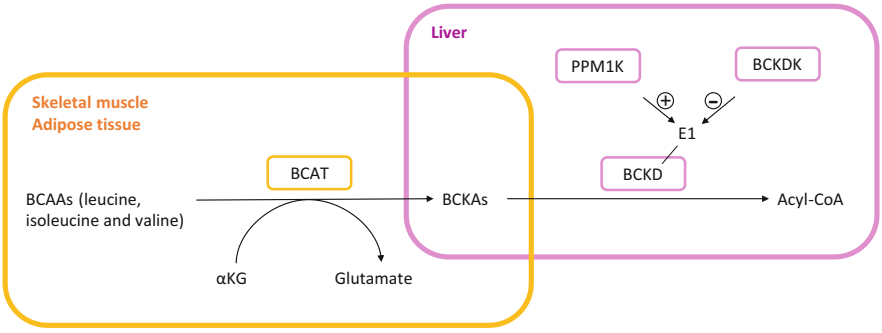

Fig. 5 The compartmentalization of BCAA catabolism. PPM1K: Protein Phosphatase, Mg^{2+}/Mn^{2+} Dependent 1 K, BCKDK: branched-chain ketoacid dehydrogenase kinase

In skeletal muscle, She et al. reported that protein abundance and activity level of both BCAT and BCKD were not different between obese and lean animals (She et al. 2007). However, White et al. observed that BCKD activity was higher in muscle of Zucker fatty rats compared to lean controls (White et al. 2016). In 2021, Lee et al. compared the global transcriptomic profile (measured by RNA-seq) of skeletal muscle between men who were overweight vs lean (Lee et al. 2021). They reported that BCAA catabolism was one of the pathways significantly different between the two groups. When looking at the specific genes, BCKD, but not BCAT, was significantly less expressed in the muscle of individuals who were overweight. Lerin et al. compared muscle transcriptome (microarray) between individuals with normal insulin sensitivity, insulin resistance, and type 2 diabetes (Lerin et al. 2016). One of the pathways correlated with insulin sensitivity was BCAA catabolism. Genes coding for BCKD, but not BCAT, contributed to this pathway annotation. Targeted gene expression measurement would be necessary to confirm the down-regulation of BCKD in human muscle in the context of obesity. Moreover, because BCKD is expressed at low levels in this tissue, the relevance of this phenomenon is not clear. The limited information available seems to indicate that BCAT in the muscle is not affected by obesity, but more studies are necessary to confirm this notion.

She et al. observed that murine genetic models of obesity had lower liver BCKD gene expression and protein abundance than lean animals (She et al. 2007). However, Kadota et al. showed that diet-induced obesity in animals results in higher, rather than lower, hepatic BCKD activity (Kadota et al. 2013). In humans, Shin et al. compared liver BCKD abundance between participants who were obese vs those who were lean (Shin et al. 2014a). Men with obesity had significantly lower abundance of BCKD and phosphorylated (active) BCKD compared to their lean counterparts. However, this obesity-related difference was not seen in women. More studies investigating BCAA catabolism in the liver of humans are required.

Obesity is associated with a downregulation of BCAA catabolism in adipose tissue, liver, and potentially skeletal muscle. Because glutamate is a by-product of the pathway, this would theoretically result in lower glutamate production and lower circulating glutamate level with (abdominal) obesity. Because we see exactly the opposite, it appears unlikely that the downregulated BCAA catabolism would be responsible for the association between glutamate and central fat accumulation.

Partial Summary

We do not yet know why circulating glutamate is positively associated with abdominal fat, but we have eliminated a few theories. First, genetics does not seem to be the main driver, because level of this metabolite show low heritability and the association between glutamate and central fat persist for a given genetic background (identical twins discordant for abdominal obesity). Dietary glutamate is unlikely to be responsible, because of a low proportion reaching circulation. Alterations in the gut microbiota could potentially be implicated, but the mechanism is not fully

elucidated. Finally, although BCAAs are also higher in obesity and their catabolism pathway involves glutamate, it is unlikely that a reduced BCAA catabolism would increase glutamate, because it is a by-product and not a substrate.

Glutamate is implicated in many pathways all over the body: transamination, anaplerosis, neurotransmitter synthesis, nitrogen handling, glutathione synthesis, and more. Abdominal fat accumulation could alter one or many of these pathways and result in increased glutamate production. Studies investigating the pathophysiology of elevated glutamate in abdominal obesity specifically are necessary to uncover this mechanism.

Steps to Be Taken Before Applications to Prognosis

Glutamate represents a potentially interesting biomarker for abdominal/visceral obesity. The value of being able to identify individuals with this condition is that it would help to focus prevention and intervention efforts. Indeed, accumulation of fat in the VAT and in other ectopic depots is very closely correlated with future cardiometabolic diseases (Tchernof and Despres 2013). Fortunately, VAT is the first fat depot to be mobilized during weight loss (Bouchard and Tremblay 1997). Therefore, glutamate could help identify people who would benefit the most from weight loss and help track the loss of this specific fat during the process. However, in order to go from potential to actual biomarker, there are many steps that still need to be completed.

The pathophysiology needs to be elucidated, at least partially. Indeed, we need to know what phenomenon is tracked by glutamate to be able to use it as a biomarker. Abdominal obesity is associated with many phenotypes, from adipocyte dysfunction to liver steatosis to aging (Tchernof and Despres 2013). The use we would make of glutamate would vary greatly depending on the mechanisms involved.

Studies suggest that individuals with high circulating glutamate levels have a higher prevalence of abdominal obesity. However, it is important to evaluate whether circulating glutamate is a better screening tool than other existing methods to identify visceral or abdominal obesity. The most commonly used tool is the WC measurement; therefore, it would represent a good benchmark to compare glutamate to. It would also be relevant to examine whether using glutamate in addition to existing measurements (e.g., WC) would increase its predicting ability.

The few studies done on circulating glutamate as a biomarker of abdominal obesity have all been done on samples mainly composed of Caucasian individuals. Large studies on more diverse populations are necessary to determine whether the association between glutamate and central fat is seen in all ethnic groups. Along the same lines, samples in the studies on glutamate were mainly composed of women. Although the limited results we have indicate no difference between sexes (Maltais-Payette et al. 2019), this still warrants further investigation.

In almost all studies mentioned in this chapter, the concentration of glutamate in plasma was measured by mass spectrometry. This is the gold standard method to measure many metabolites, but it is expensive and time consuming. If glutamate

were to be used in a clinical context, a simpler, faster, and more affordable measurement tool would be needed. There are commercial kits available on the market designed to measure glutamate, but their accuracy would have to be compared with mass-spectrometry measurements in a context of abdominal obesity.

Overall, although glutamate has great potential as a biomarker of abdominal obesity, many steps still need to be taken before it can be used in a clinical context. One of the greatest challenges is to uncover the pathophysiology underlying this association, in order to understand whether glutamate really tracks abdominal fat per se, or another concomitant phenomenon.

Mini-Dictionary of Terms

Bariatric surgeries: Procedures altering the digestive system with the intent of inducing weight loss. Some surgeries are restrictive only (gastric banding, sleeve gastrectomy), whereas others are both restrictive and malabsorptive (roux-en-y gastric bypass, bilipancreatic diversion with duodenal switch).

Gut microbiota: All microorganisms living in our digestive tract. This system interacts with the human body regarding digestion and regulation of immunity and metabolism.

Metabolomics: The comprehensive study of small molecules in a given biological sample, most often blood or urine.

Nonalcoholic fatty liver diseases (NAFLD): A range of diseases characterized by the accumulation of triglycerides in hepatocytes and a number of morphological and cellular alterations; it includes simple steatosis, steatohepatitis, and fibrosis.

Ectopic fat: Accumulation of triglycerides in lean organs such as the kidneys, skeletal muscle, heart, kidneys, and pancreas.

Metabolic syndrome: A combination of anthropometric and metabolic features that is associated with increased risk of type 2 diabetes and cardiometabolic diseases. It is defined by the NCEP-ATPIII committee as the combination of more than three of the following features: elevated WC, elevated triglycerides, high blood pressure, elevated fasting glucose, and low HDL-cholesterol.

Anaplerosis: The biochemical process responsible for replenishing the tricarboxylic acid cycle by synthesizing its intermediates from amino acids.

Key Facts

- Glutamate is a nonessential amino acid, meaning it is easily synthetized by the body.
- Glutamate is implicated in a large number of metabolic processes, notably the transamination of other amino acids, nitrogen handling, anaplerosis, and neurotransmitter synthesis.
- Circulating glutamate level is positively associated with measurements of central fat accumulation such as WC, trunk fat, and VAT area.

- Plasma glutamate is also correlated with the prevalence of T2D, CVD, and NAFLD.
- The pathophysiology underlying these associations is not elucidated, but scientific literature suggests that genetics and dietary glutamate intake are unlikely to be important contributors.

Summary Points

- Scientific investigation from the last decade has demonstrated that obesity is accompanied by changes in the plasma concentration of many amino acids.
- Glutamate has been demonstrated to be strongly associated with the amount of fat accumulated in the visceral and abdominal regions. This is of importance because this pattern of fat accumulation is highly predictive of cardiometabolic diseases. Accordingly, circulating glutamate levels have also been shown to be elevated in individuals with T2D, NAFLD, and CVD.
- The pathophysiology of this association between circulating glutamate and abdominal obesity has never been formally investigated. Twin studies indicate that genetics is likely not the main factor. Mechanistic studies have demonstrated that dietary glutamate does not reach the bloodstream in sufficient amounts to increase its concentration in abdominal obesity.
- Although circulating glutamate is a potentially interesting biomarker for visceral/abdominal obesity, some aspects need to be investigated further before it can be used as such. This include elucidating its pathophysiology, testing on more diverse populations, measuring its added value compared to or in addition to existing tools, and testing/developing simple and affordable measurement protocols.

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Leptin as a Biomarker in Nutrition and Metabolism

47

Applications to Diabetes

Heba Sadek Kassab

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Abstract

Obesity and its associated metabolic diseases became a major health problem and leading eventually to cardiovascular diseases and increased morbidity and mortality. Visceral obesity is metabolically active secreting substances called adipokines. Of them, leptin is the prototypical adipokine exerting a variety of metabolic effects. Since its discovery in 1994, leptin has been extensively studied in animals and humans, and everyday there are new data related to leptin, and

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more is still to be discovered. Leptin has a pleotropic effect involving metabolism, inflammation, obesity, immunity, diabetes, and reproduction. Higher leptin levels than normal lead to tissue insensitivity to leptin referred to leptin resistance. Diabetes, obesity, insulin resistance, and leptin resistance are closely related. This review aimed to discuss the effect of leptin on metabolism, obesity, type 2 diabetes and its complications and vice versa, and its therapeutic implementation.

Keywords

Leptin · Leptin resistance · Diabetes · Obesity · Inflammation · Immunity · Insulin resistance · Visceral obesity · Adipokines · Hyperleptinemia

Abbreviations

BBB	Blood brain barrier
BMI	Body mass index
CNS	central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
HIV	Human immunodeficiency virus
IL6	Interlukin 6
JAK-STAT	Janus kinase-signal transducer and activator of transcription
K ATP	Potassium-adenosine triphosphate
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic peptide-1
NFkB	Nuclear factor kappa B
PAI-1	Plasminogen activatorinhibitor-1
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
TNFα	Tumor necrosis factor alpha

Introduction

Obesity and diabetes prevalence is increasing worldwide. Insulin resistance is a result of obesity, especially visceral obesity, and is associated with other metabolic disorders like dyslipidemia, diabetes, and hypertension. All these are risk factors of atherosclerosis (Eckel et al. 2005).

Visceral adipose tissue produces metabolically active substances called adipokines. These substances can regulate gene expression and function in macrophages, endothelial cells, and arterial smooth muscles so they can affect the vessel wall atherogenic environment. Adipokines include many substances such as adiponectin, leptin, tumor necrosis factor alpha (TNFα), resistin, plasminogen activator inhibitor-1(PAI-1), monocyte chemotactic peptide-1(MCP-1), and free fatty acids (Lau et al. 2005).

Leptin is the prototypical adipocytokine. It was discovered in 1994. It is synthesized in white adipose tissue. Its plasma level is proportional to fat mass. Leptin has many functions including weight control, energy homeostasis, and regulation of hematopoietic, reproductive, and immune functions. The wide distribution of leptin receptors all over the body supports its pleotropic effect (Fig. 1). Leptin performs multiple functions linking metabolism, nutrition, reproduction, and immune system. During the previous 2 decades, leptin was thoroughly studied, but there is still more to be known about it (Pérez-Pérez et al. 2017, 2020).

Leptin level is elevated in obesity and its associated metabolic disorders. This may be due to leptin resistance in those individuals. Its effect extends beyond body weight regulation to include antilipogenic and glucose-lowering effect. Leptin exerts both central and peripheral effects on glucose metabolism and is affected by activation of insulin-signaling pathways. Leptin is considered a proinflammatory factor that affects insulin resistance and obesity performing the link between obesity and cardiovascular

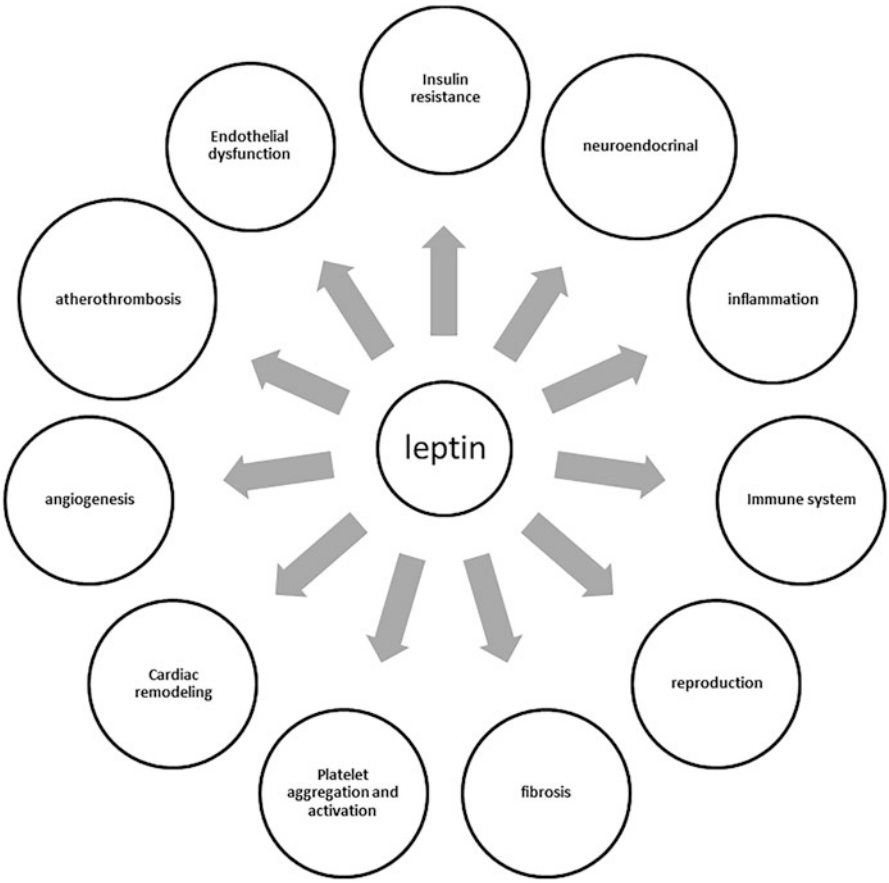

Fig. 1 Pleotropic effect of leptin. Leptin affects various functions in the body, and its receptors are distributed in many organs

disease. So, leptin could be considered as an emerging potent modulator of metabolism of glucose, proteins, and fat (Kang et al. 2020; Pereira et al. 2021).

This chapter discusses the role of leptin in metabolism, diabetes, and its complications.

Leptin

Leptin is a 16-kDa peptide hormone with 167 amino acids. It is encoded by the Lep OB gene (Zhang et al. 1994).

Leptin is synthesized mainly in white adipose tissue, and it has a circadian rhythm with the highest level at night. Plasma leptin level ranges from 1 to 15 ng/ml. This level is affected by the body adipose mass as it reflects the stored energy in adipose tissue. Thus, hyperleptinemia occurs in obese individuals and leads to loss of the normal circadian rhythm while in fasting and losing weight conditions plasma leptin level decreases (Maffei et al. 1995; Saad et al. 1998).

Leptin mRNA is also expressed in other tissues including the pituitary, hypothalamus, placenta, stomach, and mammary gland (Bado et al. 1998).

The main function of leptin is regulation of body weight hence the name "leptin" which is derived from the word *"leptos"* that means thin in Greek. Leptin gene transcription controls its rate of release. Leptin level in circulation is stable from meal to meal, but its response to metabolic stimuli may take hours (Zhao et al. 2020).

Leptin release is stimulated by high insulin and glucocorticoid levels, but on the other hand, activation of adrenergic receptors of sympathetic nervous system suppresses leptin secretion (Caron et al. 2018).

Leptin Receptors and Signaling Pathways

There are six isoforms of leptin receptors (LepRa-f) with less-understood functions. They are encoded by *db* gene, and they are members of cytokine class one family. They have the same leptin-binding domain with different intracellular domains. Some isoforms were suggested to be related to leptin transport while others can bind to leptin inhibiting its transfer (Wada et al. 2014).

The isoform responsible for leptin specific effects is LepRb which leads to activation of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Mice lacking LepRb were found to be severely obese with dyslipidemia, insulin resistance, and more liable to develop diabetes. STAT 3 is phosphorylated by JAK2 and then transferred to nucleus to play a role in leptin gene transcription (Davis et al. 2010; Iqbal et al. 2020).

Other recent pathways are also involved in leptin action. This includes another JAK2-dependent, but STAT3-independent, pathway which action is still not well established, but it is important for leptin effect. Mitogen-activated protein kinase (MAPK) is another pathway by which lipoprotein assembly is done by lepRa (Barnes et al. 2020).

Hyperleptinemia and Leptin Resistance

As mentioned before, hyperleptinemia occurs in obese individuals. Despite the state of hyperleptinemia, it is unable to decrease body weight and food intake that refers to leptin resistance. Leptin resistance could not be reversed by increasing the level of circulating leptin; this is unlike insulin resistance which could be improved with insulin administration (Caron et al. 2018).

However, it is not clear whether hyperleptinemia is a cause or a consequence of obesity. The imbalance between energy intake and expenditure will lead to increase adipose tissue mass and obesity with subsequent elevation of circulating leptin level. This elevation is proportionate to adipose tissue mass irrespective to individual's body mass index (BMI). This supports the hypothesis that hyperleptinemia is a consequence of obesity and not a cause of obesity or its associated disorders (Ogus et al. 2003).

On the other hand, hyperleptinemia is considered a predictor of cardiovascular outcome progression. Hyperleptinemia also leads to enhanced age-related adipose mass and increased liability to diet-induced obesity and insulin resistance even within normal leptin levels. These findings suggest that even slight elevation of serum leptin level is obesogenic and triggers these metabolic alterations. Moreover, certain hormones (insulin and glucocorticoids) and inflammatory mediators (TNFα and lipopolysaccharide) increase leptin level and also form a bridge between obesity and its associated disorders (Zhao et al. 2019).

Interestingly, metabolic alterations like hypothermia, hyperinsulinemia, and hyperglycemia were not detected in subjects with leptin deficiency. This could be explained by, in humans, obesity is associated with leptin target tissues insensitivity to leptin-specific defects rather than these defects themself (Kilpelainen et al. 2016).

Mechanism of Leptin Resistance

Leptin resistance arises from different mechanisms including genetic, molecular, and functional changes leading to abnormalities in molecule structure, its blood brain barrier (BBB) transport, and changes in leptin receptors and signaling pathways (Fig. 2) (Gruzdeva et al. 2019).

Genetic mutations of leptin genes (OB and DBU genes) are extremely rare in humans so they are not the main cause of leptin resistance in humans. If present, they will lead to hypothalamic hypogonadism, early obesity, and hyperphagia (Wabitsch et al. 2015).

Alteration of leptin transport through BBB is suggested to be another mechanism of leptin resistance. Excessive serum leptin levels (higher than 25–30 ng/mL) decrease BBB permeability to leptin resulting in lower cerebrospinal fluid (CSF) leptin levels. In a previous study, leptin level was lower in CSF than in blood in obese individuals but not in normal weight people. This contributes to obesity and leptin resistance (Holtkamp et al. 2004; Lee et al. 2017).

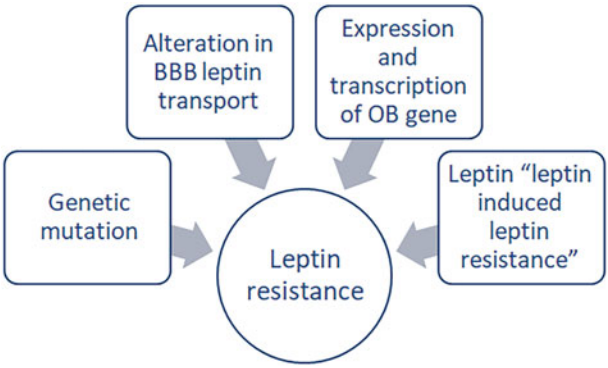

Fig. 2 Mechanism of leptin resistance. Leptin resistance arises from different mechanisms including genetic, molecular, and functional changes

The OB gene transcription also plays a role in development of leptin resistance by affecting leptin concentration and leptin-related effects. The expression of OB gene is correlated to the size of adipocyte and its lipid content. Moreover, leptin expression is affected by various external stimuli including circadian rhythm, overeating, and hunger (Zhang and Ren 2016).

Another important factor contributing to leptin resistance is leptin itself "leptin induced leptin resistance."It is a vicious circle that starts by leptin resistance which increases susceptibility to diet-induced obesity which furthermore increases leptin resistance (Gonzalez-Carter et al. 2016).

Despite all these theories of the cause responsible for leptin resistance, there is a need for further study to clarify its pathogenesis and diagnostic criteria.

Leptin and Metabolism

There is a link between insulin resistance, leptin resistance, and obesity. Hyperinsulinemia as a result of insulin resistance may potentiate leptin resistance which in turn leads to obesity and subsequently metabolic syndrome (Lustig et al. 2004) (Fig. 3).

The effect of leptin in the pathophysiology of diabetes and obesity is as important as that of insulin. Leptin plays a central role in glucose and lipid metabolism by both central and peripheral effects (Fig. 4). This effect starts at a central level where glucose uptake is modulated by leptin in brain astrocytes. In addition, glucose sensing by neurons is mediated through central leptin. However, the role of leptin in controlling lipid metabolism centrally is not yet well established (Sohn et al. 2016).

The effect of leptin on blood vessels either beneficial or deleterious is conducted through its serum level where physiological levels have a therapeutic effect while pathological levels are proatherogenic similar to obesity and diabetes (Raman and Khanal 2021).

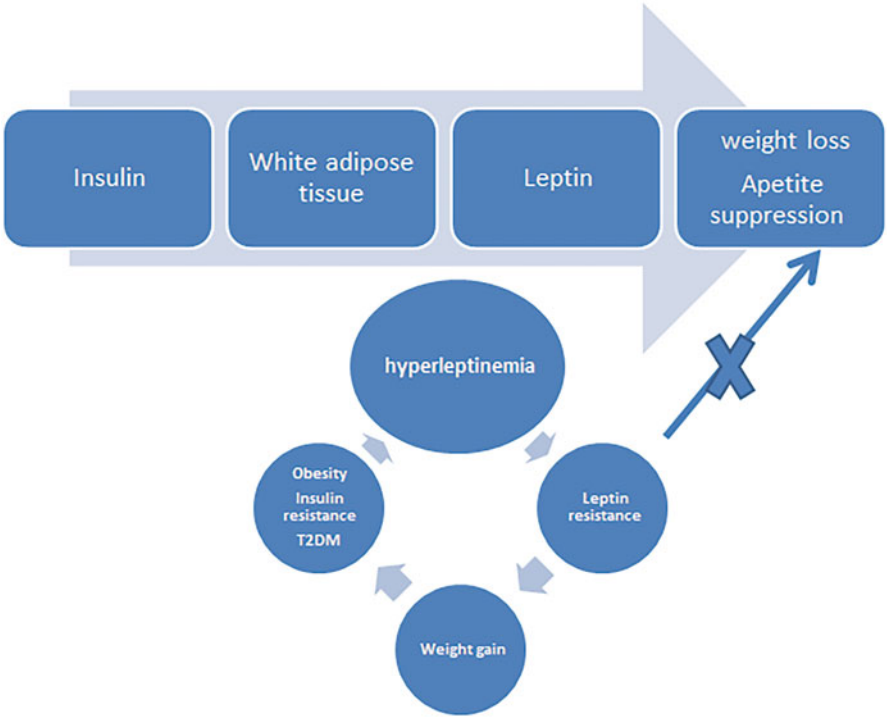

Fig. 3 Relation between leptin, obesity, and insulin resistance. Leptin secretion from white adipose tissue is stimulated by insulin. Leptin will lead to decrease in appetite and consequently weight loss. This high leptin level will lead to decrease in insulin secretion. In the presence of obesity and insulin resistance, a higher leptin level is present and leptin resistance will develop leading to tissue insensitivity to leptin and eventually weight gain

Pancreatic effects of leptin include decreasing insulin secretion from beta cells by activation of K ATP channels and also reducing glucagon secretion from alpha cells in conditions of hyperglycemia (Wu et al. 2017).

Hepatic effects of leptin include decreasing gluconeogenesis and hepatic triglycerides content and consequently reducing plasma triglycerides level. Moreover, leptin promotes inhibition of hepatic glucose production through an insulin-dependent mechanism leading to improvement in insulin resistance. As previously mentioned, leptin participates in lipoproteins assembly in the liver from stored lipids (Iqbal et al. 2020).

Leptin and Inflammation

Obesity and inflammation are interacting through adipokines. Fat deposition produces a suitable microenvironment for immune system. It was reported that low-grade inflammation is induced by application of high-fat diet with increased

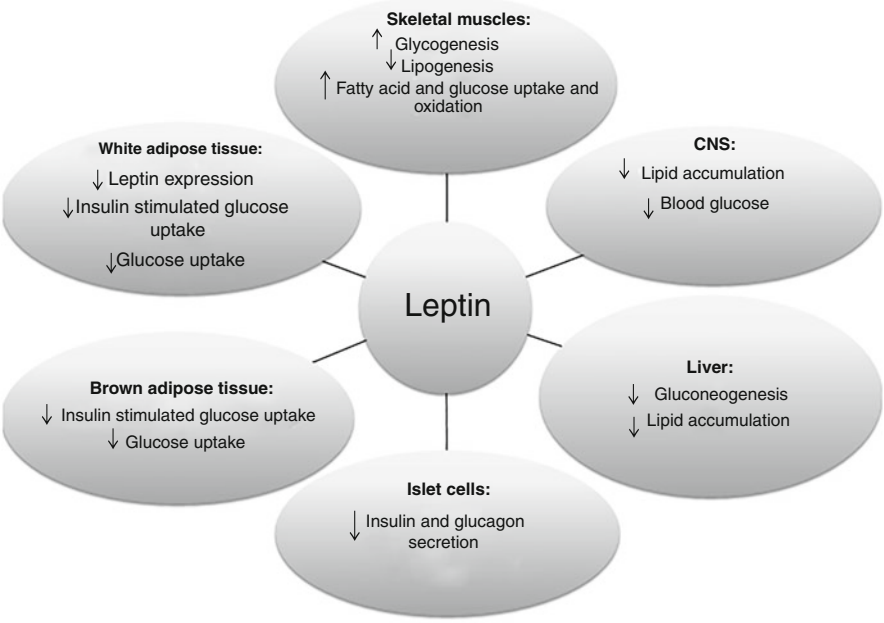

Fig. 4 The role of leptin in glucose homeostasis. Leptin regulates glucose homeostasis through its effect on different organs peripherally (liver, pancreas, skeletal muscles, and adipose tissue) and centrally

secretion of inflammatory cytokines like IL6 and TNFα. Leptin makes a link between neuroendocrine and immune system, and it is considered as an acute phase reactant with higher levels in sepsis and fever (Mattace Raso et al. 2013; Koç et al. 2003).

Inflammation is an essential regulator of body homeostasis. Any abnormality in inflammatory pathways may trigger various diseases. Although still not fully understood, the relationship between leptin and inflammation is bidirectional. Leptin is a proinflammatory mediator through increasing TNFα and NFkB. It also mediates activation of immune system (innate and adaptive) performing a link between immune system and metabolism. Leptin increases the release of reactive oxygen species, increases chemotaxis, and enhances the phagocytic function of macrophages and monocytes and the natural killer cells cytotoxic ability (Pérez-Pérez et al. 2020).

On the other hand, obesity and its associated disorders induce a state of low-grade inflammation and lead to elevation of leptin level and promoting leptin resistance through affecting leptin-signaling pathways. This leptin resistance makes the fuel for augmentation of further inflammatory response (Tilg and Moschen 2006).

Leptin is also linked to immunity and infection. Studies revealed higher leptin level in sepsis survivor patients than nonsurvivors. Also lower leptin levels were detected in HIV patients suggesting a role of reduced leptin level in

immunodeficiency. These observations highlight leptin regulation of systemic immunity and its potential future therapeutic role in infectious diseases. Moreover, leptin is suggested to contribute to vaccination strategies as it is an endogenous substance that will elicit a proper inflammatory response without side effects (Tschop et al. 2010; Estrada et al. 2002).

Obesity and higher leptin levels were related to increased morbidity and mortality from severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) infection. The determinant of the pathogenesis of viral response and disease course is the interaction between the host immune response and the virus. Hyperleptinemia and obesity are associated with impaired immune response and hence increased chemokines secretion compromising host defense against virus (Rebello et al. 2020).

Leptin and Diabetes

Leptin and Type 2 Diabetes

The pathogenesis of type 2 diabetes (T2DM) is mediated mainly through insulin resistance. Additionally, low-grade inflammation was proposed to play an important role in the development of T2DM. A number of inflammatory biomarkers were elevated in T2DM and obesity including C-reactive protein (CRP), IL6, and TNFα while decreasing body weight will improve insulin resistance and decrease the level of these mediators. These findings suggest the role of inflammation in the development of T2DM. Moreover, inflammation will lead to endothelial dysfunction which represents the central pathological mechanism of atherosclerosis and its related cardiovascular disease (DeFronzo 2004).

Leptin is thought to play a role in atherosclerosis as leptin receptors were found in atherosclerotic lesions. Another evidence is that in spite of obesity in leptin-deficient mice, they do not develop atherosclerosis, but it is still not proved that leptin or its resistance mediates atherosclerosis (Sweeney 2010).

Leptin is the regulatory adipokine of the pathways linked to inflammation-induced insulin resistance. Moreover, leptin deficiency plays a crucial role in the development of severe insulin resistance leading to disruption in adipocytes and adipose tissue and eventually lipodystrophy. Leptin effect on blood glucose level is mediated centrally through its direct central nervous system (CNS) effect and peripherally through normalization of glucagon level. This will lead to reduction in blood glucose level and decrease gluconeogenesis genes expression. When leptin is combined with insulin therapy, it leads to decrease in lipid transcription factors resulting in reducing cholesterol level (Meek and Morton 2016).

Although leptin therapy improves insulin sensitivity, reduces body weight and plasma lipids level, these effects are not found in obese patients with T2DM. In those patients, leptin administrations slightly improve HbA1c level but not insulin sensitivity. This may be due to the state of hyperleptinemia present in these patients and inducing leptin resistance. This is a turning point in research field where trying to improve leptin sensitivity rather than administration of exogenous leptin may

improve glycemic and lipid parameters in patients with T2DM (Mittendorfer et al. 2011; D'souza et al. 2017).

Recently, molecular characterization of leptin and T2DM was studied and revealed shared genetic architecture between both suggesting an underlying molecular mechanism linking leptin level and T2DM (Wang et al. 2020).

Leptin and Diabetic Complications

Previous studies reported higher incidence of obesity, endothelial dysfunction, metabolic syndrome, and hypertension in patients with T2DM with high leptin level. In this sense, leptin level was positively correlated to carotid intima media thickness and also with the incidence and severity of silent myocardial infarction. Furthermore, higher leptin level in patients with T2DM was linked to increased cardiovascular risk, cardiac autonomic neuropathy, and the occurrence of microvascular complications (Katsiki et al. 2018).

As stated previously, leptin plays an important role in inflammation and endothelial dysfunction which are the central pathological mechanisms of diabetic microvascular complications. In patients with T2DM, higher serum leptin level was correlated with neuropathy and albuminuria but not with retinopathy (Rodríguez et al. 2016).

Moreover, in another study, serum leptin level was positively correlated with urinary albumin to creatinine ratio, retinopathy, and peripheral neuropathy and negatively correlated to estimated glomerular filtration rate (AbdEl Aaty et al. 2020). Few other studies highlighted the relation between leptin and microvascular complications where most of them showed positive correlations (Yassin et al. 2019; Jung et al. 2014; Uckaya et al. 2000) while one study showed no association (Sari et al. 2010).

In patients with T2DM and normal weight, higher serum leptin level is found to be a risk factor of diabetic nephropathy. On the other hand, these correlations were not present in overweight and obese patients with T2DM. This may be due to saturation of leptin receptors in the kidney or due to insensitivity of receptors to leptin due to leptin resistance (Huang et al. 2021).

Leptin and Antidiabetic Medications

As mentioned earlier, leptin is closely linked to insulin resistance and T2DM. Whether antidiabetic medications affect leptin level or serum leptin level affects their mechanism of action and biological effects is still a rich area of research. Only few researches studied this relation in humans (Nar & Gedik 2009; Negrotto et al. 2016; Tarkun et al. 2010).

Metformin therapy leads to decrease in leptin level in patients with T2DM, improvement of hypothalamic sensitivity to leptin, and also decreased leptin level in women with polycystic ovary syndrome (a condition associated with insulin resistance) (Nar & Gedik 2009).

Sitagliptin (a dipeptidyl peptidase inhibitor) was found to reduce leptin level in humans (Li et al. 2017).

Table 1 Effect of antidiabetic medications of serum leptin level in patients with T2DM

Drug	Effect
Metformin	Decreases leptin level Improves hypothalamic sensitivity to leptin Decreases leptin level in women with polycystic ovary syndrome
Pioglitazones	Decreases leptin level
Liraglutide	Decreases leptin level
Sitagliptin	Decreases leptin level

Other antidiabetic medications like pioglitazones and liraglutide also decrease serum leptin level while sodium glucose cotransporter 2 inhibitors have no human data regarding leptin levels. As observed, most of these medications work through decreasing insulin resistance or reducing body weight which are common pathways with leptin actions. Further studies are needed to clarify this relationship (Kanoski et al. 2015) (Table 1).

Clinical Application of Therapeutic Leptin Administration

In patients with lipodystrophy, leptin therapy improves glycemic and lipid parameters by reducing HbA1c, glucose, and insulin levels. It also decreases total serum cholesterol, triglycerides, and LDL- cholesterol. Leptin also decreases hepatic lipogenesis in these cases. Moreover, metabolomics analysis of blood of patients with lipodystrophy revealed increased amino acid catabolism and fatty acid oxidation (Baykal et al. 2020).

Leptin deficiency could result from genetic mutations, defect in leptin receptors, or defect in leptin-signaling pathways. These abnormalities will alter glucose metabolism and will lead to development of obesity. Leptin administration in these cases will improve these metabolic effects with reduction in body weight, improvement of glycemic and lipid parameters by reducing plasma insulin and glucose levels and increasing insulin sensitivity. It could also reverse hepatic steatosis (Clément et al. 2018).

Moreover, leptin deficiency was found in type 1 diabetes (T1DM) patients, and leptin administration in these patients showed initial promising results (Mittendorfer and Klein 2014) (Fig. 5). Although there was no change in glycemic and lipid parameters, insulin requirement and weight were reduced significantly upon leptin administration in these patients. These findings reflect the insulin-sensitizing effect of leptin, but further studies are still needed to identify the exact role of leptin administration in T1DM (Vasandani et al. 2017).

Applications to Prognosis of Type 2 Diabetes and Its Complications

In this chapter, the relation between leptin level and T2DM and its complications was reviewed. Plenty of data studied leptin and obesity and its associated disorders (diabetes and insulin resistance) in animals, but we focused in this chapter on human

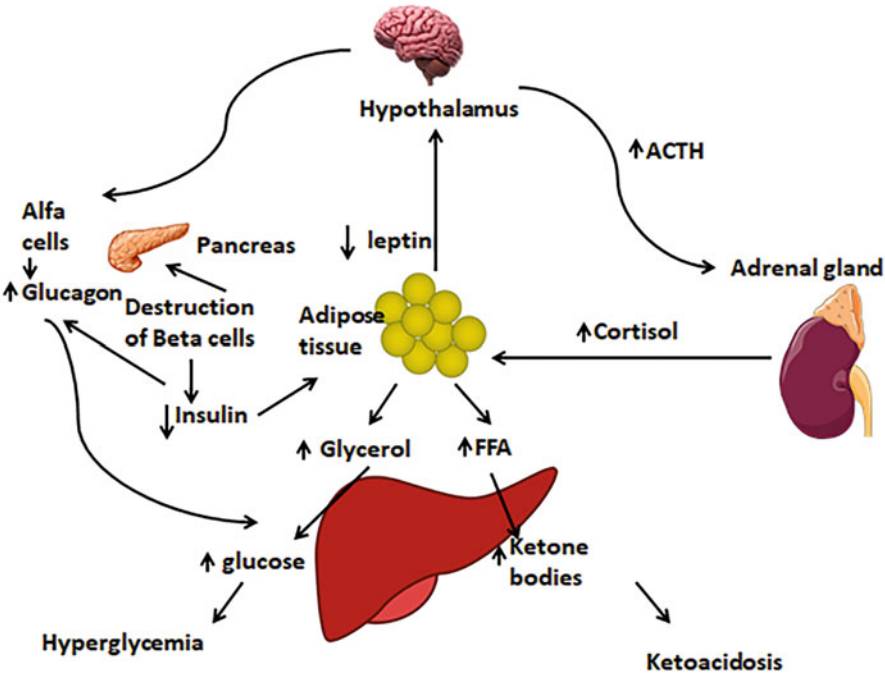

Fig. 5 Role of leptin deficiency in the pathogenesis of type 1 diabetes. In type 1 diabetes, autoimmune destruction of pancreatic beta cells leads to decreasing insulin level and consequently decreasing leptin secretion from adipose tissue. Then a cascade of effects including multiple organs and hormones leads eventually to hyperglycemia and ketoacidosis

data. Most of the previous studies reported higher leptin level in patients with T2DM and diabetic complications (Rodríguez et al. 2016). This could be beneficial in detection of patients with higher risk of developing complications and their progression. It also raised the potential role of reducing leptin resistance in improving these complications. This chapter also reviewed the relation between leptin and antidiabetic medications. Higher leptin levels were detected in patients receiving drugs improving insulin resistance and body weight (metformin, sitagliptin, pioglitazones, and liraglutide) which may share a common mechanism of action with leptin while this is still a rich area of research (Nar and Gedik 2009; Li et al. 2017; Kanoski et al. 2015).

Application to Type 1 Diabetes

Most of the researches reviewed in this chapter were discussing the relation of leptin to obesity, insulin resistance, and T2DM. There is lack of data regarding T1DM. The only clinical application of leptin in T1DM till now is significant reduction in insulin dose and body weight upon leptin administration although no significant

improvement in glycemic and lipid parameters were detected. More studies are needed to clarify the effect of leptin in patients with T1DM (Vasandani et al. 2017).

Mini-Dictionary of Terms

- Adipokines: a group of substances secreted by adipose tissue and having an endocrine function
- Chemotaxis: migration of cells in response to chemical stimuli
- Hyperphagia: abnormal increased eating
- Hypogonadism: a condition when there is decreased secretion of sex hormones due to decreased gonads function
- Leptin resistance: tissues' insensitivity to circulating leptin
- Lipodystrophy: a condition characterized by abnormal body fat amount and distribution
- Proatherogenic: promotes atherogenic activity

Key Facts of Leptin Resistance

- Obesity leads to elevation of leptin level.
- Hyperleptinemia is associated with tissues insensitivity to leptin "leptin resistance."
- Leptin resistance is associated with other metabolic disorders like obesity, insulin resistance, and type 2 diabetes.
- Improving leptin resistance is more beneficial than exogenous leptin administration in improving metabolic parameters.
- Higher leptin levels were detected in patients with diabetic complications highlighting the role of leptin resistance in the pathogenesis of these complications.

Summary Points

- Leptin is closely related to obesity, insulin resistance, and diabetes.
- Normal leptin levels have beneficial metabolic effects while abnormal levels are proatherogenic.
- Obesity and type 2 diabetes are associated with hyperleptinemia and leptin resistance.
- Higher leptin levels are associated with diabetic complications.
- The relation between leptin level and antidiabetic medications is still unclear and needs further studies.
- Therapeutic administration of leptin is only beneficial in few conditions (lipodystrophy, type 1 diabetes, and leptin deficiency).

- Improving leptin sensitivity rather than administration of exogenous leptin will improve metabolic consequences.

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Nutritional Indicators and Illness

48

The Nutrition Risk in the Critically Ill Score

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Abstract

Among patients admitted in the intensive care units (ICU) the prevalence of malnutrition is high, often associated with poor prognosis. Approximately two-thirds of critically ill patients do not meet the energy intake goals. In 2011, analyses of multicenter cohorts led to the development of the NUTrition Risk in the Critically ill (NUTRIC) score, the first malnutrition screening tool validated with data from critically ill patients. The NUTRIC score is calculated based on a variety of routinely used variables in the ICU setting, enabling the identification of patients who would benefit from aggressive medical nutrition therapy (MNT). Its modified version, the mNUTRIC, leaves out the assessment of interleukin-6 without compromising its prognostic ability. In the more recent version (NUTRIC-SF), it is combined with frailty and sarcopenia screening tools to reflect the novel malnutrition criteria. All scores emphasize on the provision of macronutrients, with increased scores being associated with 28-day mortality, increased ICU length of stay, and duration of mechanical ventilation support. Compared with other malnutrition screening tests, the NUTRIC scores have a higher prognostic value and a lower number needed to screen (NNS), being the only tools validated in the ICU setting.

Keywords

Critical illness · ICU · Malnutrition · Mortality · Number needed to screen · Sarcopenia · Metabolic adaptation · Frailty · Protein requirements · Energy deficit · Length of stay

Abbreviations

APACHE II	Acute Physiology and Chronic Health Evaluation II
ASPEN	American Society for Parenteral and Enteral Nutrition
BMI	Body mass index
BW	Body weight
CFS	Clinical frailty scale
COVID-19	Coronavirus disease 2019

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EN	Enteral nutrition
GLIM	Global Leadership Initiative on Malnutrition
ICU	Intensive care unit
IL-6	Interleukin-6
IOI	Incidence of infection
LBM	Lean body mass
LOS	Length of stay
MNT	Medical nutrition therapy
mNUTRIC	Modified NUTrition Risk in the Critically ill
MUST	Malnutrition Universal Screening Tool
NUTRIC	NUTrition Risk in the Critically ill
NUTRIC-SF	NUTrition Risk in the Critically ill – Sarcopenia Frailty
RCT	Randomized controlled trial
SARC-Calf	Strength, assistance with walking, rising from a chair, climbing stairs, and falls with calf circumference
SARC-F	Strength, assistance with walking, rising from a chair, climbing stairs, and falls
SGA	Subjective global assessment
SMART	Specific, Measurable, Achievable, Relevant, and Time-bound
SOFA	Sequential Organ Failure Assessment

Introduction

Nosocomial malnutrition is one of the most important predictors of poor disease prognosis, increased length of stay (LOS), mortality, and reduced functional ability among hospitalized patients (Foo et al. 2021). Research indicates that malnutrition at admission is often missed, whereas, when developed post-admission, it is rarely diagnosed (Kirkland and Shaughnessy 2017), despite the variety in the available screening tools.

In the intensive care unit (ICU) in particular, where the severity of medical problems is heightened, with most patients experiencing life-threatening, critical conditions, nutritional status and disease prognosis are synchronized. A wide variation (38–78%) has been reported regarding the prevalence of malnutrition among the critically ill patients, depending on the screening tool used (Lew et al. 2017). On the other hand, the aim of medical nutrition therapy (MNT) inside the ICU setting is to avoid the development of malnutrition in previously well-nourished patients while preventing further worsening of the nutritional status of previously malnourished individuals (Hill et al. 2021). Malnutrition in the ICU is associated with increased LOS and need for readmission, as well as many poor clinical outcomes, including incidence of infection (IOI) and risk of hospital mortality (Alberda et al. 2009; Lew et al. 2017).

The mechanistic drivers of ICU malnutrition are multifactorial, stemming from the increased caloric requirements due to metabolic stress, infection or trauma, and the reduced dietary intake as a result of appetite decrease, both of which are resulting in a progressive energy deficit (Casaer and Van den Berghe 2014). The latter is

worsen by immobilization, inflammatory, and endocrine stress responses paired with the frequently prolonged mechanical ventilation, all increasing susceptibility to muscle wasting, cachexia, and weakness (Casaer and Van den Berghe 2014; Dixit et al. 2021). During the past decades, ICU feeding practices have evolved, as a consequence of landmark randomized controlled trials (RCTs) and systematic reviews (Dixit et al. 2021).

Metabolic Response to Acute Injury/Trauma and Timing of Feeding in the ICU

In a multinational study, Heyland revealed that the great majority of critically ill patients, including those classified as being of high nutritional risk, fail to receive an adequate amount of dietary intake (Heyland et al. 2015). In further detail, it appears that on average, 38.8 h pass after admission until the feeding of critically ill patients is initiated. The mean amount of energy received corresponds to 61.2% of the prescribed dose, and the provision of protein reaches 57.6% of the actual patient needs, leading to 74.0% of the critically ill patients with unmet energy requirements (<80% of energy goal) (Heyland et al. 2015).

Iatrogenic underfeeding consists of a common practice in the ICU setting, based on the distinct metabolic phases of acute critical illness and the physiological responses to stress. According to an autobiographical note by Sir Cuthbertson (2016), during the first phase of acute illness (known as the ebb or early shock phase), hemodynamic instability is apparent, inducing hormonal changes, aiming to prioritize the delivery of energy substrates to the tissues (Lambell et al. 2020). The ebb phase is characterized by an increase in endogenous glucose production and a concomitant reduction in the energy expenditure, both aiming to prolong survival when feeding is not feasible. In the second phase (the flow phase), tissue is broken down, providing substrates aiming to fulfil the needs for the "fight or flight" response, while reducing the risk of infection and bleeding (Lambell et al. 2020). Thereafter, a convalescent (anabolic) phase is initiated, with resynthesis of lost tissue (Sobotka and Soeters 2009). MNT is adapted to these phases aiming to provide the appropriate amount of nutrients required in each stage, without compromising prognosis.

According to Lambell (Lambell et al. 2020), approaches on the dogma of "one size fits all" or "set and forget" do not fit the ICU setting. Although critically ill patients are heterogeneous, in many of them disease prognosis may improve from the provision of adequate MNT (Hill et al. 2021). Energy deficit in the ICU is associated with increased LOS, infectious complications, and mortality (Alberda et al. 2009). Early meta-analyses comparing early (within 24 h of ICU admission) versus delayed enteral nutrition (EN) initiation suggested a plethora of improved outcomes following the first, including a reduced LOS, mortality, infection, and pneumonia risk (Heyland et al. 2003; Doig et al. 2009). These results guided the 2016 American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines (McClave et al. 2016), recommending the prompt initiation of feeding following admission to ICU. However, meta-analyses of RCTs revealed either uncertainty, or a

lack of difference between early versus delayed EN initiation and risk of mortality within 30 days, pneumonia, feed intolerance, or gastrointestinal complications (Tian et al. 2018; Padilla et al. 2019).

Malnutrition Definition, Screening, and Diagnosis

For several years, malnutrition was an umbrella term, covering any nutrient deficit, with an emphasis on inadequate energy and protein consumption (Wischmeyer 2011). Traditional markers, including the body mass index (BMI) or serum albumin/prealbumin concentrations, have limited value in evaluating nutrition risk (Zhang et al. 2021a). The lack of a single anthropometric or analytical value that could be predictive of malnutrition alone led to the development and proposition of several diagnostic criteria, until an international consensus was reached (Jensen et al. 2009). According to the recently developed Global Leadership Initiative on Malnutrition (GLIM) criteria (Cederholm et al. 2019), a combination of phenotypic and etiologic criteria is evaluated when determining a malnutrition diagnosis, including percentage of body weight (BW) loss, low BMI and muscle mass, reduced food intake or assimilation, and inflammation.

Moreover, screening tools are employed to identify the risk for malnutrition and the subsequent need for more aggressive nutritional care. In their majority, these tools were developed and validated in outpatient or ambulatory settings and are not ICU-specific (Anthony 2008). On the other hand, the NUTrition Risk in the Critically ill (NUTRIC) score was first developed in 2011, using a multicenter sample of ICU patients and providing the paradigm shift in the nutritional assessment and the provision of ICU nutritional care (Heyland et al. 2011).

With malnutrition diagnosis being a difficult task in the critically ill (McClave et al. 2016), the latest ASPEN guidelines introduced nutrition assessment in the ICU setting (Sheean et al. 2020), given that BMI is universally acknowledged as a crude estimate of nutritional status. Moreover, bedside nutrition assessment techniques are not sensitive and specific enough to detect changes in lean body mass (LBM) (including cachexia and sarcopenia) taking place during the metabolic adaptations to injury/trauma (Bhuachalla et al. 2018). Given however that the exact losses in LBM are required for the calculation of protein requirement, and that muscle wasting is associated with increased inflammation in the critically ill (Witteveen et al. 2017), emphasis was recently placed on the use of body composition (Sheean et al. 2020).

Development of the NUTRIC, mNUTRIC, and NUTRIC-SF Scores and Components

The aim of the NUTRIC score is to quantify nutritional risk in the critically ill and identify patients most likely to benefit from aggressive MNT (Heyland et al. 2011). Aggressive MNT makes use of goals using the SMART (Specific, Measurable, Achievable, Relevant, and Time-bound) principles (Nakahara et al. 2021). The

tool is based on a conceptual model balancing starvation (energy deficit), inflammation, nutritional status, and clinical outcomes (Heyland et al. 2011).

Variables were selected for the tool based on their model fit and their routine use in clinical practice. The initial tool included a variety of parameters affecting the conceptual model's balance, including age, Acute Physiology And Chronic Health Evaluation II (APACHE II) score (Knaus et al. 1985), Sequential Organ Failure Assessment (SOFA) score (Ferreira et al. 2001), the plethora of existing comorbidities, days in hospital to ICU admission, and interleukin-6 (IL-6) concentrations (Fig. 1). The score ranges between 0 and 10 with higher scores being indicative of a higher risk for 28-day mortality and lower scores being associated with better prognosis.

Although the circulating concentrations of pro-inflammatory cytokines, like the IL-6, are increased as a trauma-stress response, they are not routinely assessed in most

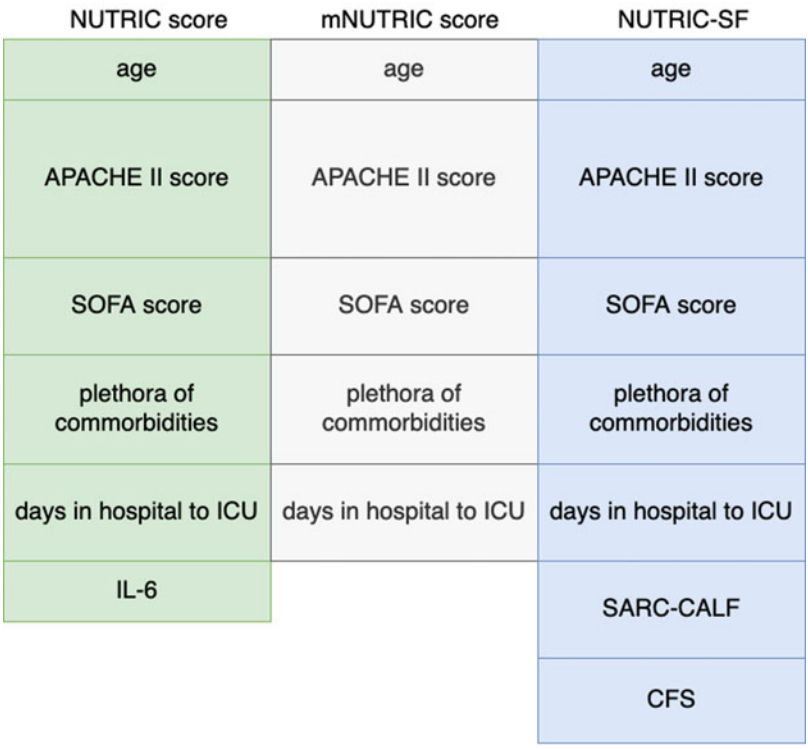

Fig. 1 NUTRIC, mNUTRIC, and NUTRIC-SF components. APACHE II, Acute Physiology and Chronic Health Evaluation II (Knaus et al. 1985); CFS, clinical frailty scale (Rockwood et al. 2005); ICU, intensive care unit; IL-6, interleukin-6; mNUTRIC, modified NUTrition Risk in the Critically ill (Rahman et al. 2016); NUTRIC, NUTrition Risk in the Critically ill (Heyland et al. 2011); NUTRIC-SF, NUTrition Risk in the Critically ill – Sarcopenia Frailty (Lee et al. 2021); SARC-F, Strength, assistance with walking, rising from a chair, climbing stairs, and falls; SARC-Calf, SARC-F combined with calf circumference (Barbosa-Silva et al. 2016); SOFA, Sequential Organ Failure Assessment (Ferreira et al. 2001)

of the ICUs. This created a bottleneck for internists wishing to apply the NUTRIC score to their patients. To correct for this issue, the authors of the NUTRIC score opted in excluding the IL-6 assay, producing the modified-NUTRIC (mNUTRIC) score (Rahman et al. 2016) (Fig. 1). The mNUTRIC was also validated and proved to have a good predictive value in prioritizing critically ill patients most likely to benefit from optimal macronutrient provision (Rahman et al. 2016). The score ranges between 0 and 9 with ascending scores suggesting a greater risk for 28-day mortality.

Recently, the GLIM criteria and the introduction of body composition in the ASPEN guidelines for the critically ill changed the landscape of malnutrition assessment in the ICU. For this, the mNUTRIC was adapted to reflect these new requirements, by incorporating valid tools to detect LBM changes. Thus, the mNUTRIC was combined with one tool assessing sarcopenia (strength, assistance with walking, rising from a chair, climbing stairs, and falls [SARC-F] combined with calf circumference [SARC-Calf]) (Barbosa-Silva et al. 2016) and one evaluating frailty (clinical frailty scale [CFS]) (Rockwood et al. 2005) to improve its prediction regarding adverse outcomes and identify patients in need for early optimal nutrition in the ICU (Lee et al. 2021). The produced tool was named NUTRIC-SF (Fig. 1).

Use of the NUTRIC/mNUTRIC Scores and Clinical Outcomes

Since their development, the scores have been applied in a variety of critically ill patients, including mechanically ventilated burn patients (Alfonso Ortiz et al. 2021), patients with end-stage liver disease (Mayr et al. 2020), patients with coronavirus disease 2019 (COVID-19) (Zhang et al. 2021b), cirrhotic patients with acute gastro-esophageal variceal bleeding (Tsai et al. 2019), patients with sepsis (Hung et al. 2019; Jeong et al. 2019; Tsai et al. 2021), patients with neurological problems (Zhang et al. 2021a) or who have undergone cardiothoracic surgery (Zheng et al. 2021), etc. According to a recent meta-analysis (Ibrahim et al. 2020) of 4076 critically ill patients, a high mNUTRIC score is associated with an increased risk of 28-day mortality, increased ICU LOS, and longer duration of mechanical ventilation.

Moreover, the use of these scores has been embraced by researchers globally, with several translations and adaptations to other languages and many more uses globally, as evidenced by relevant publications (Reis et al. 2019).

Comparison of the NUTRIC Score to Other Screening Tools for Nutritional Risk

Several studies have compared the NUTRIC score to other tools of similar aim. Compared with the Malnutrition Universal Screening Tool (MUST), the NUTRIC demonstrated a higher sensitivity in assessing the need for early MNT in critically ill patients (Hameed and Harris 2017). However, the MUST has not been validated for use in the ICU setting. Subsequently, Majari showed that within the ICU, the MUST

failed to associate with longer LOS, prolonged mechanical ventilation, and 28-day mortality (Majari et al. 2020).

When the subjective global assessment (SGA) was applied as a complimentary tool to the NUTRIC score, patients with a score ≥ 4 for nutritional risk (based on the SGA) exhibited a 6-time higher 28-day mortality risk compared with those without nutritional risk, indicating that the complementarity of the 2 tools was high (Gonzalez et al. 2019). This finding was further verified in a Brazilian study (Oliveira et al. 2020). Moreover, a lower number needed to screen (NNS) (Rembold 1998) was calculated for patients with a high NUTRIC score and SGA compared with patients with a NUTRIC <4 (Gonzalez et al. 2019). The specificity and the Youden index (Ruopp et al. 2008) of the NUTRIC appears to decrease with each increasing threshold (from 3 to 5), whereas, in parallel, the sensitivity is increased (Gonzalez et al. 2019). When the SGA and the NUTRIC were compared, out of 114 patients (100%) classified as being at high SGA risk in total, 40 (35%) exhibited low and the remaining 74 (65%) high NUTRIC scores, respectively (Aguila et al. 2018).

In an Iranian study of 440 critically ill patients, the area under the curve for predicting 28-day mortality was 0.806, 0.695, and 0.551 for the mNUTRIC, Nutritional Risk Screening (NRS)-2002 score (NRS-2002), and MUST tools, respectively (Majari et al. 2020).

The findings mentioned above collectively indicate that the NUTRIC scores are the most appropriate ones for critically ill patients; however, they can be complemented with the SGA to improve their prediction value.

Limitations of the NUTRIC and mNUTRIC Scores

One of the limitations of the scores consists of the lack of classical "dietary" data such as recent food intake and change in body weight (Lee and Heyland 2019). However, weighing critically ill patients is not always feasible, in particular in the ICU setting (Hill et al. 2021).

Moreover, dietary data cannot always be obtained as many patients are admitted in the ICU during coma or while being unconscious; thus, a diet history cannot be obtained. Nevertheless, this can also be an advantage, as the use of the scores is not limited to experienced nutritionists or dietitians, but is available to anyone working in the ICU setting with access to the NUTRIC parameters of each patient. Other researchers argued that the score focuses on the administration of macronutrients, mainly protein and energy, failing to reveal patients who might benefit from the supplementation of pharmaconutrients (e.g., antioxidants or glutamine) (Ibrahim et al. 2020).

According to de Vries, an important limitation stems from the fact that the score was developed and validated using the exact same database, thus limiting its external validity (Heyland et al. 2011; Vries et al. 2018). Furthermore, during its development, the nutritional history and practices post-ICU admission were only suboptimally accounted for (Ibrahim et al. 2020).

Conclusions

MNT of the critically ill patient is important for prognosis and survival. Nutrition assessment is a prerequisite for the set of accurate energy and protein goals. Incorporation of body composition methods can aid malnutrition diagnosis. The NUTRIC score is a tool validated in the ICU setting, which can provide useful information for the nutritional status of the patients and the recognition of those who would benefit the most from MNT.

Applications to Prognosis

Application of the NUTRIC score can help in identifying ICU patients who are in nutrition risk and need nutritional care (Heyland et al. 2011). Most importantly, within the content of an ICU, either NUTRIC or mNUTRIC scores can be applied to predict the "hard" outcome of the 28-day mortality rate (Heyland et al. 2011; Rahman et al. 2016). Clinically meaningful outcomes, such as lowered LOS and duration of mechanical ventilation, were associated with the NUTRIC/mNUTRIC scores (Heyland et al. 2011; Ibrahim et al. 2020). These scores seem to have a better prognostic value compared with other common tools, such as the MUST and the NRS-2002 (Majari et al. 2020). Alternatively, the complementary use of the NUTRIC score with the SGA can be applied in the ICU setting (Gonzalez et al. 2019).

Applications to Other Diseases or Conditions

In this chapter, the applicability of the NUTRIC score was assessed for critically ill patients. Patients in the ICU experiencing malnutrition have increased energy requirements and experience muscle waste and cachexia. Conditions which are metabolically demanding or prone to increased malnutrition risk have been studied with the NUTRIC scores. Examples include burns, end-stage liver disease, cirrhosis, sepsis, cardiothoracic surgeries, neurological problems, and COVID-19 (Hung et al. 2019; Jeong et al. 2019; Tsai et al. 2019, 2021; Mayr et al. 2020; Zheng et al. 2021; Alfonso Ortiz et al. 2021; Zhang et al. 2021a). However, recording of NUTRIC scores misses assessments of dietary intake, changes in BW, and the provision of immunonutrients, such as glutamine (Lee and Heyland 2019; Ibrahim et al. 2020).

Mini-dictionary of Terms

Cachexia: Ongoing muscle loss due to an underlying illness.

Ebb phase: Early hypometabolic stage in response to acute trauma injury.

Energy deficit: The provision of lower energy compared with the goal required to maintain energy balance.

Malnutrition: A state of over- or undernutrition leading to deficiencies, excesses, or imbalances in the dietary intake (including either energy or nutrients).

Number needed to screen: The number of patients screened to prevent one death.

Sarcopenia: Loss of skeletal muscle mass and function.

Trophic feeding: The provision of minute volumes of enteral feeds (insufficient to meet caloric goal) aiming to stimulate the gastrointestinal tract.

Key Facts of the NUTRIC Scores

- Clinical features of malnutrition in the intensive care units (ICU) include muscle wasting, cachexia, and weakness.
- Mechanistic drivers of malnutrition in the ICU include increased caloric requirements, reduced dietary intake, immobilization, inflammation, and endocrine stress responses.
- Underfeeding in the ICU can lead to a first phase of reduction in the energy expenditure.
- The first phase of the physiologic response to underfeeding (the ebb phase) is the decrease of energy expenditure along with the increase in the glucose production.
- The second phase (the flow phase) includes the "fight or flight" response: the breakdown of tissue so that essential substrates are available to cover immediate needs.
- Subsequently, an anabolic phase follows with regeneration of the lost tissue.
- In all malnutrition stages, medical nutrition therapy (MNT) can be adapted to accommodate the individual needs.
- Application of the NUTRIC screening tool and its modified versions assigns a score with higher values indicating malnutrition risk and suggesting the initiation of MNT in malnourished patients.

Summary Points

- Malnutrition in the ICU is multifactorial, due to increased energy requirements and reduced dietary intake, both of which are producing a progressive energy deficit.
- The recent diagnostic criteria for malnutrition proposed by the GLIM initiative redefined malnutrition according to phenotypic and etiologic indicators: somatometric measurements, food intake, inflammation, and disease burden.
- The NUTRIC scores emphasize on the provision of macronutrients, mainly protein and energy, while leaving out immunonutrients, such as glutamine.
- Medical nutrition therapy (MNT) in the ICU aims in avoiding the development of malnutrition in previously well-nourished patients while preventing further worsening of the nutritional status of previously malnourished individuals.

- MNT is adapted to the three metabolic phases of acute injury/trauma aiming to provide the appropriate amount of nutrients required in each stage, without compromising prognosis.
- Energy deficit in the ICU is associated with increased length of stay (LOS), readmission to the ICU, infectious complications, and mortality.
- The NUTrition Risk in the Critically ill (NUTRIC) score identifies critically ill patients who will benefit the most from aggressive MNT.
- The NUTRIC score accounts for the age of the patient, the APACHE II and SOFA score, the plethora of existing comorbidities, days in hospital to ICU admission, and interleukin-6 (IL-6) concentrations.
- The NUTRIC score ranges between 0 and 10, with greater scores indicating a higher risk for 28-day mortality and lower scores being associated with better prognosis.
- The modified NUTRIC (mNUTRIC) score (without the IL-6) has been validated and has a good predictive value in prioritizing critically ill patients most likely to benefit by optimal macronutrient provision.
- The mNUTRIC score ranges between 0 and 9, with higher scores suggesting a greater risk for 28-day mortality.
- The NUTRIC-SF is a combination of the mNUTRIC, the SARC-F, and the CFS, aiming in introducing body composition assessment in patients with malnutrition admitted in the ICU.

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The Importance of Inflammatory State in Vitamin Supplementation Studies

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How to Deal with the Biomarkers

Bahareh Nikooyeh and Tirang R. Neyestani

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Abstract

Inflammation is a natural immune response to the foreign invaders to the body. However, inflammatory reaction needs to be precisely orchestrated from initiation

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to termination to keep the tissues and organs of the body safe. Consequently, any defect in mounting inflammatory response or in regulating its termination could be in opposition with the survival of the organism. Chronic inflammation-associated disorders are the most prevalent pathologies and the main causes of death in the world. Among these disorders, cardiovascular disease (CVD), diabetes, chronic obstructive pulmonary disease (COPD), and autoimmune disorders have the highest occurrence globally. Continuous attempts have been making to reinforce the antioxidant defense systems through increased intake of naturally occurring antioxidants in fruits and vegetables or vitamin supplementation to suppress augmented inflammatory response and the resulting oxidative stress. In this chapter, the most recent evidence of the reciprocal effects of inflammation and vitamins will be reviewed. Finally, some important remarks for the future studies will be presented.

Keywords

Inflammation · Vitamins · Supplementation · Inflammatory biomarkers · Cytokines · CRP

Abbreviations

ABCA1	ATP-binding cassette transfer A1
APC	Activated protein C
APP	Acute phase proteins
APR	Acute phase response
ARIC	Atherosclerosis Risk in Communities
CFA	Complete Freund's adjuvant
CLP	Cecal ligation and puncture
COPD	Chronic obstructive pulmonary disease
Covid-19	Coronavirus disease of 2019
COX	Cyclo-oxygenase
CRP	C-reactive protein
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
FM	Fat mass
Gas	growth arrest-specific
GGCX	γ-glutamyl carboxylase
GLA proteins	γ-carboxyglutamic acid proteins
GRP	Gla-rich protein
HMGB1	High-mobility group box 1
hs-CRP	High-sensitivity C-reactive protein
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-gamma
IL	Interleukin
LBP	Lipopolysaccharide-binding protein
LOX	Lipoxygenase

LPS	Lipopolysaccharide
LT	Leukotrienes
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
MGP	Matrix Gla protein
NCDs	Noncommunicable diseases
NF-κB	Nuclear factor kappa-B
NHANES III	Third National Health and Nutrition Examination Survey
Nrf2	Erythroid 2-related factor 2
NSCLC	Nonsmall cell lung cancer
OS	Oxidative stress
PAI	Plasminogen activator inhibitor
PAMPs	Pathogen-associated molecular patterns
PGE_2	Prostaglandin E_2
PIVKA	Protein induced by vitamin K absence/antagonism
PKC	Protein kinase C
PLP	Pyridoxal-5′-phosphate
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRRs	Pattern recognition receptors
RA	Retinoic acid
RAR	Retinoic acid receptor
RBP	Retinol binding protein
ROS	Reactive oxygen species
T2D	Type 2 diabetes
TAC	Total antioxidant capacity
TGF	Transforming growth factor
Th	T-helper
TNF	Tumor necrosis factor
t-PA	Tissue plasminogen activator
Treg	Regulatory T cell
TTR	Transthyretin
VAD	Vitamin A deficiency
VCAM	Vascular cell adhesion molecule
VDD	Vitamin D deficiency
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
α-TTP	α-tocopherol-transfer protein

Introduction

During those years when infectious diseases were uprooting and suppressing human communities, less people might have thought that some serious diseases were caused indeed by nutritional deficiencies and not infectious agents. The life-changing

findings of James Lind, a Scottish surgeon in Royal Navy, on the miraculous effect of lemon and lime juice against scurvy in 1753 (Whitehead 1987) and of Joseph Goldberger on curing effect of animal and leguminous protein foods on pellagra during 1915–1926 (Berdanier 2019) unveiled the impact of nutrition on human health. These findings were a tremendous stimulus for chemists to find out the nature of these vital elements. Among them, Casimir Funk has undoubtedly a unique place. He was working on one of such elements whose deficiency, as shown by Christiaan Eijkman, would cause a lethal neurosis in chickens (lately named thiamine) and found nitrogen in it. Hence, he coined these "vital amines" as "vitamine" (Piro et al. 2019). This was a very fundamental step in the path of discovery of the causes and treatment of many vitamin deficiency disorders (Piro et al. 2019). Though vitamin deficiencies have not been eradicated yet (Amrein et al. 2020; World Health Organization 2009; Allen 2009; Rowe and Carr 2020), the health-promoting effects of vitamin supplements have been the focus of more attention since a few decades ago (Wallace 2015). Among the interested health aspects, inflammatory status has probably been the most investigated.

In this chapter, after a brief review of the pathophysiology and laboratory methods for evaluation of inflammation, the effect of inflammation on the vitamin status and the influence of vitamin supplementation on inflammatory biomarkers are reviewed using the latest available evidence. Finally, some remarks for future studies are made.

Inflammatory Response: Good, Bad, and Ugly

The major task of the immune system is recognizing "self" from "non-self" and mounting a reaction to the latter (Gonzalez et al. 2011). Inflammation is, therefore, a natural immune response to the foreign invaders to the body. However, inflammatory reaction needs to be precisely orchestrated from initiation to termination to keep the tissues and organs of the body safe. Consequently, any defect in mounting inflammatory response or in regulating its termination could oppose with the survival of the organism.

Depending on the time course, inflammation may be categorized as acute, subacute, and chronic. Among these, acute inflammation is more familiar and sensible as everybody has experienced it. It may occur immediately following an infection or injury and may last for up to a week usually accompanying with heat (calor), redness (rubor), swelling (tumor), pain (dolor), and immobility or loss of function (function laesa) (Hannoodee and Nasuruddin 2021). These presentations are due to release of several soluble mediators including acute phase proteins, cytokines, and chemokines that recruit macrophages, neutrophils, and other immune cells to the place. Subacute inflammation, lasting from 2 to 6 weeks, occurs during a period wherein acute inflammation is turning to chronic. Finally, chronic inflammation has a much longer period lasting for months, years, and occasionally lifelong (Hannoodee and Nasuruddin 2021). Though all three kinds of inflammation can be either localized or systemic, trauma usually evokes a local inflammatory response whereas systemic inflammation occurs in response to infections or multiple trauma (Brøchner and Toft 2009). Apart from these inflammatory responses, commonly

attended by medical care, there are conditions causing a so-called "chronic low-grade systemic inflammation" that is not necessarily accompanied by a physical presentation but may predispose for several noncommunicable diseases (NCDs) (Castro et al. 2017; Furman et al. 2019). A growing body of evidence suggests a crucial role for nutritional status, notably percent of body fat mass (FM), and diet in low-grade systemic inflammation (Minihane et al. 2015). Fortunately, reducing weight and especially visceral fat is commonly accompanied by subsidence of this kind of endogenous systemic inflammation (Sarin et al. 2019).

Altogether, inflammatory response is necessary for survival (good). However, chronic inflammation persisting for weeks or months usually needs medical assistance (bad). Finally, in certain circumstances severe acute inflammation can be lethal (ugly) (Belabed 2020).

Pathophysiology of Inflammation

Following recognition of "non-self," inflammation is the main manifestation of the immune defense mechanism triggered by innate immunity whose function is to eliminate the invading pathogens, damaged cells, or irritants and thereby to protect "self" tissues and organs (Ashley et al. 2012). The initial response of the immune system to a harmful stimulus is through acute inflammation which involves the expansion of blood vessels (vasodilation), increased blood flow, and capillary permeability and chemotactic movement of granulocytes toward inflammation site through capillary walls (diapedesis) (Pahwa et al. 2020). The immune cells residing in the involved tissue mainly dendritic cells, histiocytes, macrophages, mast cells, and Kupffer cells initiate inflammatory response via their surface receptors known as pattern recognition receptors (PRRs). PRRs can bind two sets of compounds, i.e., pathogen-associated molecular patterns (PAMPs), that are originated from pathogens, and damage-associated molecular patterns (DAMPs), that are from injured host cells (Gasteiger et al. 2017). Upon interaction of PRP with PAMP or DAMP, the immune cell will be activated thereby releasing cellular mediators that cause clinical features of inflammation including heat, swelling, and pain (hyperalgesia). Several circulating proteins are also involved in these reactions including complement system and clotting factors (Amarante-Mendes et al. 2018).

These events are orchestrated through myriad of cellular receptors and mediators including cytokines. These cellular mediators may have either proinflammatory or anti-inflammatory function. Among proinflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α have especially been associated with several inflammatory human diseases including osteoarthritis (Kapoor et al. 2011) and ischemic stroke (Pawluk et al. 2020). On the other hand, IL-4, IL-5, IL-10, IL-13, IL-33, and transforming growth factor (TGF)-β are among anti-inflammatory cytokines that inhibit chronic activation of the immune cells, and hence they were proposed as a therapeutic target in such chronic inflammatory diseases as rheumatoid arthritis (Chen et al. 2019). The secretion of these cytokines is regulated at molecular level by several transcription factors. Nuclear factor kappa-B (NF-κB), for instance,

is a regulator of both innate and adaptive immune responses and has an axial role in inflammatory reactions by upregulating several proinflammatory cytokine genes and participating in regulation of inflammasomes (Liu et al. 2017), the cytosolic multiprotein oligomers of the innate immunity that induce inflammatory reactions (Guo et al. 2015). On the other hand, peroxisome proliferator-activated receptor gamma (PPAR-γ), also known as the glitazone receptor, or NR1C3 encoded by the PPARG gene, is a type II nuclear receptor that can modulate inflammatory reactions through its anti-inflammatory actions (Villapol 2018).

Chronic inflammation-related disorders are the most prevalent pathologies and the main causes of death in the world. Among these disorders, cardiovascular disease (CVD), diabetes, chronic obstructive pulmonary disease (COPD), and autoimmune disorders have the highest occurrence globally. Just in Iran, for instance, the prevalence of CVD has dramatically increased during last four decades causing 46% of all mortalities (Sarrafzadegan and Mohammmadifard 2019).

Inflammation-Induced Oxidative Stress

In major traumas and systemic inflammations, following a short period of decreased metabolism (up to 24 h), there may be a hypermetabolic state (Brøchner and Toft 2009). The hypermetabolic state is accompanied with upregulation of several mitochondrial genes including mitochondrial-specific proteases (LONP1 and CLPP) and mitochondrial translocases (TIM23, TIM17B, and TOM40) and generation of ample amounts of free radicals including reactive oxygen species (ROS) (Ogunbileje et al. 2016). Usually, there are antioxidant defensive systems to inactivate these free radicals. It is noteworthy that production of free radicals phylogenetically is a primitive defense mechanism which also occurs during several normal processes, notably cellular oxidation (Nikooyeh and Neyestani 2016). However, in certain circumstances, antioxidants may fail to counterbalance the extragenerated free radicals, the situation called oxidative stress (Nikooyeh et al. 2020). It is believed that oxidative stress (OS) is associated with several human pathologies including CVD, malignancies, neurodegenerative disorders, and diabetes (Sharifi-Rad et al. 2020). Continuous attempts have been making to reinforce the antioxidant defense systems through increased intake of naturally occurring antioxidants in fruits and vegetables or antioxidant supplementation to suppress OS (Liu et al. 2018). It is not, therefore, surprising that in many vitamin supplementation studies with the aim of suppressing inflammatory pathways, biomarkers of OS are also evaluated. However, in this chapter the effect of vitamin supplementation on OS will not be reviewed in detail.

Evaluation of Inflammatory Biomarkers

Following an infection or tissue injury, a set of metabolic changes occurs which is commonly accompanied by changes in serum concentrations of certain proteins. This process is called “acute phase response” (APR), and the reactive proteins are

Table 1 Some of the acute phase proteins (Gulhar et al. 2021)

Acute phase reactants	
Positive	Negative
C-reactive protein (CRP)	Albumin
Haptoglobin	Transthyretin (Prealbumin)
Angiotensinogen	Antithrombin
α-1 acid glycoprotein	Tansferrin
Serum amyloid A	Transcortin
Lipopolysaccharide-binding protein (LBP)	Antithrombin
Ferritin	
α-1 antitrypsin	
Hepcidin	
Fibrinogen	
Vitronectin	
Procalcitonin	

called "acute phase proteins or reactants" (APPs). The concentration of certain APPs increases (positive APP), and that of some others decreases (negative APP) in the course of inflammatory reaction (Gulhar et al. 2021). Among these, C-reactive protein (CRP) and albumin are commonly used in nutritional studies to evaluate the inflammatory state. Table 1 shows some of the APPs that commonly are evaluated in nutritional studies and assessments.

Apart from APPs, there are several laboratory tests to evaluate systemic inflammation including cytokine assays and molecular biomarkers (Macritchie et al. 2020). Depending on the level of interest of the inflammatory pathways, the profile of laboratory tests could be different.

Systemic Inflammation and Fat-Soluble Vitamins

Vitamin A and Carotenoids

The effect of vitamin A on immune function has been found long before the recognition of its chemical structure and other actions. As an immunoenhancer, vitamin A is needed for both native and acquired immune responses and for mounting inflammatory reactions. Carotenoids, on the other hand, are commonly potent antioxidants with anti-inflammatory properties.

Vitamin A

Vitamin A, a common name for a group of chemically different compounds with more or less similar functions, is an essential nutrient for normal vision, cellular differentiation, and immunity. The history of none of the essential nutrients is as much tied up to infections and inflammation as vitamin A is. Edward Mellanby and Harry N. Green, following conducting several experimental

studies, concluded that vitamin A is necessary for the immune competence hence named it an "anti-infective agent" (Semba 1999).

Effect of Inflammation on Vitamin A Status

The effect of inflammation on vitamin A status has been investigated in both animal and human studies. Inflammation may cause decreased serum concentrations of retinol, hyporetinolemia, impaired absorption, and increased urinary excretion of vitamin A (Rubin et al. 2017). During inflammation-induced APR, the concentrations of circulating vitamin A transport proteins, namely retinol binding protein (RBP) and transthyretin (TTR), decrease and consequently serum retinol concentrations also decline. Downregulation of RBP and TTR hepatic synthesis and hence reduction in serum retinol during APR occurs even before reaching CRP to its peak concentration (Rubin et al. 2017). Circulating concentrations of IL-6, the main regulator of hepatic transporters during APR (Siewert et al. 2004), inversely correlate with serum RBP and retinol concentrations (Rubin et al. 2017). Nevertheless, following subsidence of inflammation, serum retinol level is normalized indicating compartmentalization of retinol during APR. Some evidence indicates accumulation of retinol in the liver during inflammatory reaction (Gieng et al. 2005). In the studies on evaluating vitamin A status in the presence of inflammatory disease, it has been suggested to correct circulating retinol concentrations according to severity of inflammation mostly evaluated by serum CRP values (Thurnham 2015).

Effect of Vitamin A on Inflammation

Vitamin A is metabolized to retinoic acid (RA), which is a key regulator of cell proliferation and differentiation. By activating nuclear retinoid receptors, RA regulates enormous number of genes including those involved in the immunity and inflammatory pathways. In thymus and bone marrow, RA binds to nuclear retinoic acid receptor (RAR) through which regulates the expression of apoptosis genes including Bcl-2 and Fas. By this process, vitamin A is involved in the regulation of blood and immune cells population (Huang et al. 2018b). However, the overall effect of vitamin A on inflammation is the subject of debate. While some evidence indicates vitamin A has anti-inflammatory effects mainly through modulation of the immune response and maintenance of the epithelial integrity (Reifen 2002), some reports suggest a role for vitamin A in boosting inflammatory response and tissue damage via downregulating of anti-inflammatory cytokines like TGF-β (Castellani et al. 2010). Notwithstanding, in the Child Health Study of Ghana, vitamin A supplementation to under 5 children actually did not result in increased serum concentrations of APPs including α-1-acid glycoprotein, serum amyloid A, and CRP in the supplemented children as compared with the placebo group (Filteau et al. 1995). Similarly, vitamin A supplementation in 3–6 y Indonesian children had no significant effect on circulating APPs (Semba et al. 2000). Therefore, the evidence on inflammation-boosting effect of vitamin A supplementation is inconclusive. It may be worth to evaluate baseline inflammatory states of the children using determination of CRP and (if possible) IL-6 before initiation of supplementation to have a better understanding of the efficacy of the intervention (Del Giudice and Gangestad 2018).

Carotenoids

Carotenoids, also known as tetraterpenoids, are commonly plant-organic pigments in different colors including yellow, orange, and red that may also be produced by certain algae, bacteria, and fungi. They have general and specific effects on human health. All carotenoids are considered as potent antioxidants while β-carotene is also a provitamin that can be metabolized to vitamin A in the body. Lutein and zeaxanthin are found in the macular pigment of the eye (Chew et al. 2013) with protective effects against age-related macular degeneration (Wu et al. 2015). A huge body of evidence indicates a role for carotenoids in improving cognitive function and cardiovascular health and also in preventing certain types of malignancies (Eggersdorfer and Wyss 2018). A recent study reported a significant inverse association between circulating carotenoids levels and all-cause and cause-specific mortality rates (Fujii et al. 2021).

Effect of Inflammation on Carotenoids

Low-circulating concentrations of carotenoids and especially β-carotene have been reported to be linked with increased risk of overall and cause-specific mortality, notably due to cancers and CVD (Huang et al. 2018a). Controversially, most clinical trials failed to show any beneficial effect of β-carotene supplementation (Omenn et al. 1996). Even some meta-analytical studies reported an increased mortality in β-carotene-supplemented subjects (Bjelakovic et al. 2007; Bjelakovic et al. 2012). These controversial observations raised the issue that maybe the association between carotenoids (specifically β-carotene) and health is confounded by some other factors. The mechanism and importance of this inflammation-induced decrement of serum carotenoids still need to be elucidated. However, this issue must be taken into consideration in supplementation studies (Schweigert 2001). Considering the fairly variable bioavailability of carotenoids (~3–40%), which can be affected by dietary (food matrix and fats) and host factors (health and disease status, genetic variations) (Desmarchelier and Borel 2017) as well as the type of the caroteinod (Olmedilla-Alonso and Rodríguez-Rodríguez 2020), in supplementation studies in the context of inflammatory diseases the achievable circulating concentrations of the caroteinoids may be low and not necessarily corresponding to the administered dose (Rubin et al. 2017).

Effect of Carotenoids on Inflammation

The effects of carotenoids on inflammation and inflammatory diseases have been extensively investigated. Nevertheless, the results of supplementation clinical trials have been controversial. Some studies reported the effect of lycopene, a non-provitamin carotenoid naturally found in red fruits and vegetables including tomato and watermelon, on T cell-dependent adaptive inflammatory response in adults with T2D (Neyestani et al. 2007b, c), and carotenoid supplementation has been proposed for management of diabetic retinopathy (Lem et al. 2021). On the contrary, supplementation with a combination of antioxidants (including carotenoids) could not subside the inflammatory biomarkers in subjects with T2D

(Rytter et al. 2010). Along the same line, in a large clinical trial conducted on 5220 well-nourished adults, supplementation with a combination of antioxidants (vitamins C and E, beta-carotene, zinc, and selenium) for 7.5 y did not confer any significant protective effect against development of metabolic syndrome (MetS) (Czernichow et al. 2009). It is noteworthy that proinflammatory state or the so-called "low-grade systemic inflammation" has a crucial role in development of MetS and its further morbidities (Neyestani 2012). However, the inflammatory biomarkers were not evaluated in that study. Moreover, MetS results from a complex interaction between genetic and environmental factors (Pollex and Hegele 2006). It seems, therefore, oversimplistic to expect MetS be prevented just by a supplementation and without any modification of lifestyle.

A recent meta-analysis revealed the overall suppressing effect of carotenoids on inflammatory biomarkers including CRP and IL-6 (Hajizadeh-Sharafabad et al. 2021). Though the anti-inflammatory effects of carotenoids have been mostly attributed to their antioxidative properties (Kawata et al. 2018), other mechanisms including modification of intracellular signaling cascades and gene expression may also be involved. Carotenoids can downregulate NF-κB and upregulate nuclear factor erythroid 2-related factor 2 (Nrf2) (Kaulmann and Bohn 2014), a transcription factor encoded by the NFE2L2 gene that regulates the expression of certain antioxidant proteins in response to inflammation and oxidative stress (Ma 2013). Nonetheless, the proposed mechanisms can vary depending on the carotenoid type, concentration, and also the stage and severity of the inflammation (Rubin et al. 2017).

Vitamin D

Vitamin D is a lipid-soluble secosteroid. The vital role of vitamin D in calcium metabolism and maintenance of bone health has been recognized long ago. However, during the past decade, a growing body of evidence suggested several "non-calcemic functions" and significant associations of low vitamin D status with increased risk of several chronic diseases including CVD, certain types of malignancies, types 1 and 2diabetes, neurodegenerative diseases, and all-cause mortality (Lopiccolo and Lim 2010).

Humans can obtain vitamin D mainly through direct exposure to sunlight, diet, and dietary supplements. Vitamin D_3 or cholecalciferol is biosynthesized from 7-dehydrocholesterol in skin exposed to ultraviolet light. Vitamin D_2, ergocalciferol, is produced from plant sterol ergosterol and can be found in irradiated fungi and some supplements. Both isoforms of vitamin D are hydroxylated in the liver by 25-hydroxylase (CYP2R1) to produce 25-hydroxyvitamin D (25(OH)D), a reliable indicator of vitamin D status. 25(OH)D is further hydroxylated in the kidney by the enzyme 25-hydroxyvitamin D-1αhydroxylase (CYP27B1) to form the active hormone, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) or calcitriol which exerts its functions by regulating gene transcription through a nuclear high-affinity vitamin D receptor (VDR). VDR is expressed in the most cell types. It has been estimated

that vitamin D regulates some 2000 genes either directly through vitamin D response elements or indirectly via other pathways (Bikle and Christakos 2020). The most common laboratory test to assess vitamin D status is circulating 25(OH)D assay. However, different platforms may give various results. Using harmonization methods may help to lessen the inter-system differences (Nikooyeh et al. 2017).

Effect of Inflammation on Vitamin D

An increasing number of descriptive studies indicate an association between low vitamin D status and various human inflammatory diseases including CVD (Verdoia et al. 2021), diabetes (Harinarayan 2014), rheumatoid arthritis (Kerr et al. 2011), systemic lupus erythematosus (Borba et al. 2009), multiple sclerosis (Runia et al. 2012), and common infectious diseases (Watkins et al. 2015). Nevertheless, it is uncertain that decreased circulating 25(OH)D concentration is a predisposing factor or the consequence of the inflammation.

Some evidence indicates that inflammation may cause low-circulating 25(OH)D (Autier et al. 2014; Mangge et al. 2015). Inflammatory response may result in reduction of 25(OH)D via augmented oxidative stress usually seen in chronic diseases. It is likely that oxidative stress expedites biodegradation of vitamin D and interferes with the key enzymes of the biosynthesis of 25(OH)D and 1,25(OH)D (Autier et al. 2014).

The Effect of Vitamin D on Inflammation

The existing evidence suggests anti-inflammatory and immune-modulating properties for vitamin D whereby it may have alleviating effects in various inflammation-related diseases including depression (Kaviani et al. 2020), CVD (Norman and Powell 2014), diabetes (Nikooyeh et al. 2011), and autoimmune disorders (Szodoray et al. 2008). It is documented that vitamin D modulates the synthesis of TNF and IL-6 in monocytes. Also, $1,25(OH)_2D$ upregulates inhibitor of NF-κB and thereby suppresses inflammatory response by macrophages (Yin and Agrawal 2014).

A clinical correlate to this evidence is provided by a recent meta-analysis that reported supplementation with vitamin D reduces the levels of key inflammatory factors, such as high sensitivity (hs)-CRP, TNF-α, and IL-6 in patients with diabetic nephropathy suggesting that vitamin D might protect kidney functions and delay diabetic nephropathy progression (Wang et al. 2019).

Vitamin D contributes to regulation of the proliferation, differentiation, and function of the immune cells including DCs, macrophages, and T and B lymphocytes. Interestingly, these cells contain the enzyme 1-α-hydroxylase and are able to produce $1,25(OH)_2D$ locally leading to paracrine effects (Guillot et al. 2010). $1,25(OH)_2D$ is a key link between inhibition of NF-κB activation in macrophages and downregulation of monocyte chemoattractant protein (MCP-1) and IL-6 (Sanchez-Niño et al. 2012).

At the cellular level, $1,25(OH)_2D$ associates with T-helper (Th)1/Th2 and Th17/regulatory T cells (Treg) balances inducing a shift from inflammatory Th1/Th17 to the less inflammatory Th2/Treg profile. This results in decreased secretion of proinflammatory cytokines including TNF-α, interferon (IFN)-γ, IL-2, IL-12,

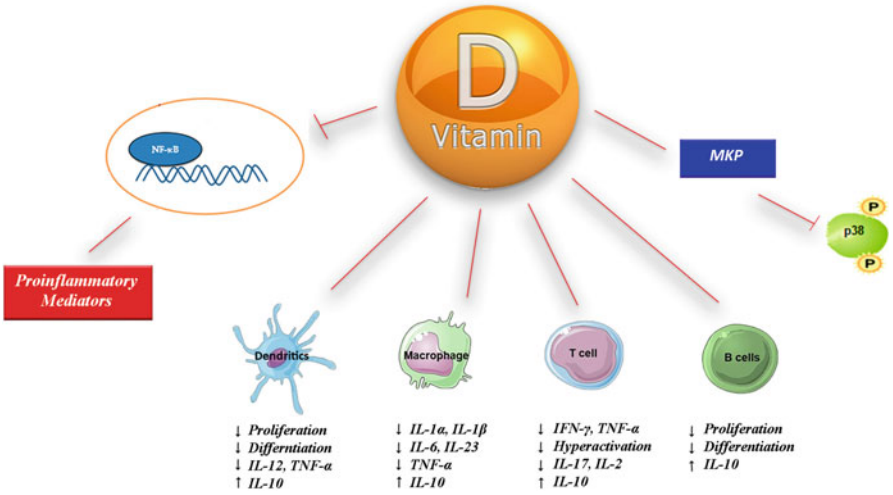

Fig. 1 Connection of vitamin D and inflammation. Vitamin D can modulate the inflammatory response via several mechanisms including effects on the interplay between dendritic cells (DCs), macrophages, and T and B lymphocytes. The effects include downregulation of proinflammatory cytokines, TNF-α and IFN-γ, and upregulation of anti-inflammatory IL-10. Proinflammatory stimuli lead to p38 MAP kinase phosphorylation and activation which subsequently induces expression of many proinflammatory proteins such as IL-6 and TNFα. 1α,25$(OH)_2$D induces MKP1 expression which dephosphorylates and inactivates p38 MAP kinase (Cristelo et al. 2021; Guillot et al. 2010)

IL-17, and IL-21 but increased production of anti-inflammatory cytokines like IL-10 (Cannell et al. 2014; Aranow 2011).

The newly emerged coronavirus severe acute respiratory syndrome (Covid-19) is characterized by excessive generation of proinflammatory cytokines, so called "cytokine storm," which has a direct link with the disease mortality (Ragab et al. 2020). Because of its modulatory action on cytokines generation, vitamin D has attracted huge attention as a potential therapeutic agent against Covid-19 (Ohaegbulam et al. 2020; Grant et al. 2020; Ranaei et al. 2021). Figure 1 shows the effects of vitamin D on inflammatory cells and mediators.

Vitamin E

Vitamin E is a common name for eight chemically diverse compounds, four tocopherols, and four tocotrienols, all with antioxidant properties (Traber and Bruno 2020). As a fat-soluble compound, vitamin E is absorbed in digestive tract along with dietary fats, so malabsorption syndromes may cause vitamin E deficiency. Both tocopherols and tototrienols occur as alpha (α), beta (β), gamma (γ), and delta (δ) forms. In the liver, α-tocopherol is bound with a high affinity to a specific protein α-tocopherol-transfer protein (α-TTP), which along with ATP-binding cassette

transfer A1 (ABCA1) integrates α-tocopherol into lipoproteins for further transportation to other tissues. The affinity of α-TTP to other E vitamers is far less so that non-α-tocopherol vitamers have a very shorter half-life due to their catabolism in the liver (Jiang 2014). Determination of circulating α-tocopherol concentration is the most common method for assessment of vitamin E status in humans (Leonard and Traber 2019).

Effect of Inflammation on Vitamin E Status

The evidence on the effect of inflammation on vitamin E is rather scarce. In a case-control study, fasting circulating concentrations of certain antioxidants including α-tocopherol, lutein, and lycopene in subjects with nonsmall cell lung cancer (NSCLC, $n_1 = 13$) were compared with those in apparently health controls ($n_2 = 22$). NSCLC subjects had augmented inflammatory response as indicated by increased serum CRP concentration. The concentrations of all measured antioxidants were significantly lower in NSCLC subjects than in controls. Also, there were inverse correlations between serum concentrations of CRP and those of antioxidants including lutein and α-tocopherol (Talwar et al. 1997). In another cross-sectional study on 100 children with malaria (50 with severe and 50 with mild malaria) and 50 healthy children as control group, the associations among circulating concentrations of α-tocopherol and several carotenoids, indicators of disease severity, APPs, and antioxidant status were investigated. It is noteworthy that dietary assessment was not performed in either of these studies. Though the reported findings may indicate the boosted utilization of vitamin E due to inflammatory response, decreased dietary intake of vitamin E and other antioxidants in the course of disease is highly plausible.

Effect of Vitamin E on Inflammation

Huge body of evidence indicates an anti-inflammatory function for vitamin E that occurs through T cell-regulatory effects and consequent downregulation of proinflammatory cytokines and prostaglandin E_2 (PGE_2), a lipid-derived mediator. PGE_2, which is produced from arachidonic acid through activation of cyclo-oxygenase (COX)-2 pathway, exacerbates inflammatory responses (Tsuge et al. 2019). Studies have demonstrated that vitamin E can inhibit COX-2 at the posttranslational level (Lewis et al. 2019). The other enzymatic pathway, 5-lipoxygenase (5-LOX), produces chemoattractant and inflammatory leukotrienes (LTs) from arachidonic acid and whereby has a major role in inflammatory diseases (Joshi and Praticò 2015). Some evidence showed that long-chain ω-carboxylate metabolites of vitamin E are potent allosteric inhibitors of 5-LOX (Joshi and Praticò 2015; Pein et al. 2018) (Fig. 2). Vitamin E can also suppress production of several pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 (Wong et al. 2019) and can alleviate systemic inflammatory response syndrome (Bulger and Maier 2003).

Vitamin E isoforms may have different effects on inflammatory pathways. It was reported that, for instance, α-tocopherol downregulates but γ-tocopherol upregulates protein kinase C (PKC)-α (Cook-Mills 2013), a serine and threonine-specific protein kinase that regulates several cellular functions including proliferation, apoptosis, and

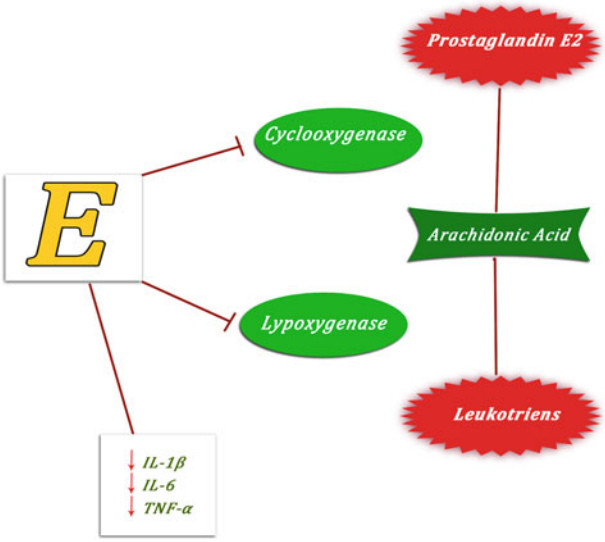

Fig. 2 Connection of vitamin E and inflammation. Vitamin E can modulate the inflammatory response via several mechanisms including downregulation of prostaglandin E2 (PGE2), which exacerbates inflammatory responses. Vitamin E can also inhibit 5-lipoxygenase (5-LOX) that produces chemoattractant and inflammatory leukotrienes. Vitamin E can suppress production of several proinflammatory cytokines including TNF-α, IL-1β, and IL-6

inflammation (Singh et al. 2017). Consequently, supplementation with different isoforms of vitamin E, namely α-tocopherol and γ-tocopherol, may bring about boosting anti-inflammatory and proinflammatory pathways, respectively (Cook-Mills 2013; Cook-Mills and Mccary 2010). Nevertheless, a meta-analytical study reported supplementation with both α-tocopherol and γ-tocopherol would result in a significant decrement in circulating CRP concentrations (Saboori et al. 2015).

Though vitamin E is considered safe even at high doses, some evidence challenges this notion. An experimental study reported increased renal tissue concentrations of MCP-1, IL-6, TNF-α, and plasminogen activator inhibitor (PAI)-1 in male mice supplemented with high doses of vitamin E (25× RDI, close to the upper tolerable limit) for 6 weeks (Jansen et al. 2016). Along the same line, a meta-analysis found a significant association between vitamin E supplementation dose and all-cause mortality rate especially for doses greater than 150 IU/D and warned not to take vitamin E supplements ≥400 mg/d (Miller Iii et al. 2005).

Vitamin K

Vitamin K, an essential fat-soluble micronutrient, refers to a family of compounds having a naphthoquinone nucleus comprising phylloquinone (K_1) and several forms of menaquinones (K_2). Phylloquinone and menaquinones are synthesized by plants

and anaerobic bacteria, respectively. Vitamin K is essential for a unique posttranslational chemical modification in a group of proteins with calcium-binding properties (called vitamin K-dependent proteins or γ-carboxyglutamic acid (Gla)-proteins). Vitamin K-dependent proteins are activated through the conversion of glutamic acid (Glu) residue of their molecules to γ-carboxyglutamic acid. This conversion is catalyzed by the γ-glutamyl carboxylase (GGCX). The process results in oxidation of vitamin K hydroquinone and production of vitamin K epoxide by vitamin K oxidoreductase in the vitamin K cycle. Vitamin K epoxide is reduced to vitamin K hydroquinone by vitamin K epoxide reductase (Nowicka and Kruk 2010) (Fig. 3).

The widely appreciated role of vitamin K-dependent proteins is in blood coagulation. The vitamin K-dependent coagulation proteins that are synthesized in the liver have either coagulant or anticoagulant properties. They include prothrombin (coagulant factor II), plasma procoagulants factors VII, IX, and X, and the anticoagulant proteins C and S. However, the beneficial role of vitamin K in health has far exceeded its function as a GGCX-dependent hepatic clotting factor in coagulation. Other vitamin K-dependent proteins which have properties other than coagulation activity such as matrix Gla protein (MGP), osteocalcin, growth arrest-specific (Gas) 6, and Gla-rich protein (GRP) have been identified (Hao et al. 2021) (Fig. 4).

Though coagulation tests, including prothrombin time, have been applied to assess vitamin K status, they do not have sufficient precision due to lack of sensitivity and specificity. Measurement of undercarboxylated prothrombin (protein induced by vitamin K absence/antagonism, PIVKA-II) is another approach indicating hepatic vitamin K status. Determination of PIVKA-II is mostly done for medical

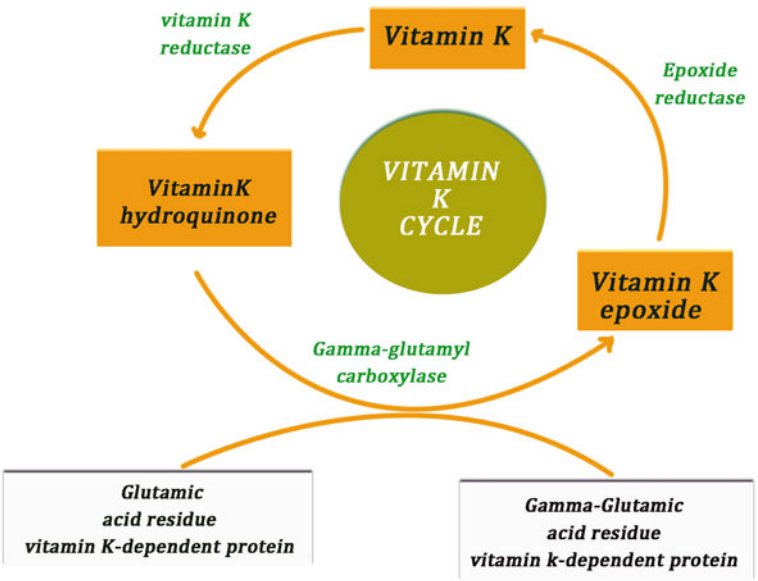

Fig. 3 Vitamin K cycle

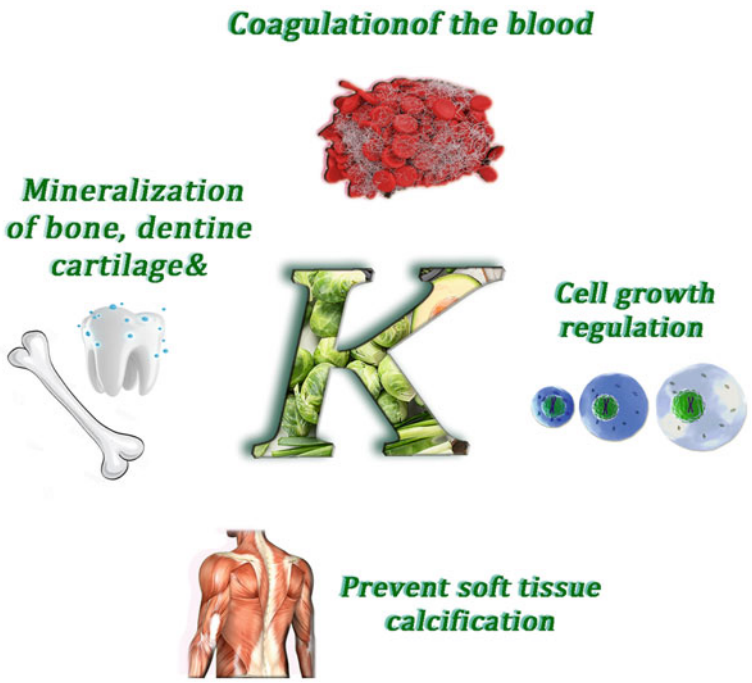

Fig. 4 Vitamin K functions

purposes including confirmation of the diagnosis of hemorrhagic disease of newborn. Circulating phylloquinone assay is the most commonly used method of assessment of vitamin K status (Card et al. 2020).

Effect of Inflammation on Vitamin K

Evidence of the effect of systemic inflammation on vitamin K status is scarce and mostly confined to descriptive association studies. In an epidemiological investigation on 1,381 subjects from Framingham Offspring Study (1997–2001, mean age = 59 y, and 52% females), a significant inverse association was observed between vitamin K status, as assessed by determination of plasma phylloquinone and phylloquinone intake, and inflammatory biomarkers including CRP. The investigators concluded that vitamin K might have an anti-inflammatory function (Shea et al. 2007). Considering the dietary sources of vitamin K are mostly green leafy vegetables, the reported associations might be secondary to the low intake of other plant antioxidants including vitamin C and carotenoids. Furthermore, the cross-sectional nature of this study makes any decisive determination of causality very difficult, if not impossible. In other words, lowered vitamin K status might be the consequence, rather than the cause, of inflammatory reaction. Along the same line, in a study on 379 apparently healthy subjects aged 60–81 y from both sexes, though initial circulating phylloquinone concentrations were inversely associated with those

of CRP and IL-6, supplementation with 500 μg/d phylloquinone for 3 years did not result in any significant changes in CRP and IL-6 concentrations (Shea et al. 2008). The effect of systemic inflammation on vitamin K must be clarified by further studies.

Effect of Vitamin K on Inflammation

A growing number of experimental and human studies suggest an inflammatory function for vitamin K. Vitamin K inadequacy has been linked with several important inflammatory-associated diseases including cystic fibrosis, inflammatory bowel disease, pancreatitis, chronic kidney disease, and osteoporosis (Kleinman and Fracchia 2010; Nakajima et al. 2011; Sikkens et al. 2013; Mccabe et al. 2013).

An experimental study using a rat model reported anti-inflammatory and cellular protective effects of vitamin K against oxidative stress during lipopolysaccharide (LPS)-induced inflammation (Ohsaki et al. 2006). This effect of vitamin K is exerted through inhibition of phosphorylation of I-κB kinase thereby nuclear translocation of NF-κB and the activation of the NF-κB pathway are suppressed (Ohsaki et al. 2010).

Some evidence indicates that vitamin K suppresses activation of the mitogen-activated protein kinase (MAPK)-signaling pathways, the major players during inflammatory responses, especially in monocytes and macrophages (Hodges et al. 2017).

Vitamin K may interfere with inflammatory reaction indirectly through vitamin K-dependent proteins. Activated protein C (APC), in addition to its anticoagulant activity, has anti-inflammatory properties by suppressing the production of pro-inflammatory cytokines (Christiaans et al. 2013). Besides, APC has an inhibitory effect on IL-6 release from LPS-stimulated human neutrophils (Galley et al. 2008), suppresses neutrophil, monocyte, and lymphocyte chemotaxis and controls expression and activation of NF-κB (Mckelvey et al. 2014).

It is likely that antioxidant and anti-inflammatory effects of vitamin K are independent of its activity as a cofactor for GGCX (Dai et al. 2020). Future studies may reveal a new preventive role for vitamin K against various inflammation-associated age-related chronic diseases (Simes et al. 2019). The anti-inflammatory mechanisms of action of vitamin K are summarized in Fig. 5.

Systemic Inflammation and Water-Soluble Vitamins

Vitamin C

Chemically, vitamin C (ascorbic acid or ascorbate; AA) is a weak sugar acid whose structure relates to glucose. Functionally, it is a water-soluble antioxidant that cannot be synthesized in human body due to lack of the functional key enzyme L-gulonolactone oxidase (Padayatty and Levine 2016).

Ascorbate is generally regarded as a reducing agent (electron donor) that can donate electrons in the redox reactions and reduce oxidizing species, or oxidants. It also acts as the cofactor of a wide array of enzymes including monooxygenases,

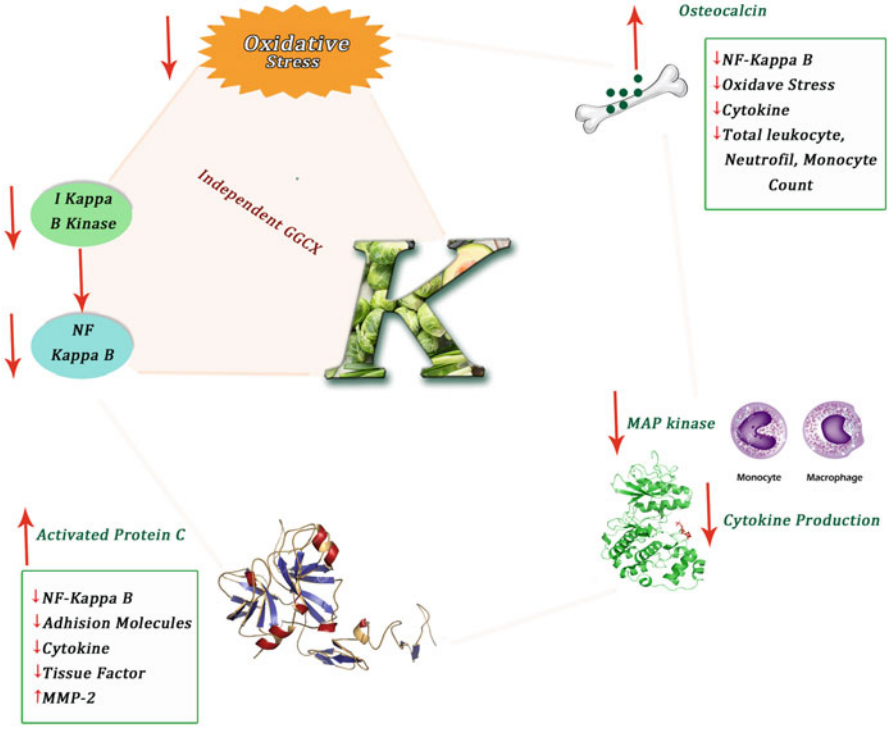

Fig. 5 Proposed mechanisms of anti-inflammatory action of vitamin K

dioxygenases, and amine oxidase (Fig. 6). Vitamin C is an important nutrient for the biosynthesis of collagen, L-carnitine, and the conversion of dopamine to norepinephrine (Padayatty and Levine 2016). Vitamin C status is assessed mostly by determination of plasma or leukocyte L-ascorbic acid concentration (Card 2019).

Effect of Inflammation on Vitamin C

Evidence of the influence of inflammation on vitamin C status is limited and mostly confined to descriptive association studies. In a cross-sectional study using National Health and Nutrition Examination Survey III (1988–1994), data on 14,519 American subjects from both sexes aged 20 y and above, an inverse association between circulating concentrations of CRP and several antioxidants including some carotenoids and ascorbic acid was found. The authors concluded that inflammation-induced oxidative stress would deplete body pools of antioxidants (Ford et al. 2003). As no data on dietary antioxidant intake was presented, it is plausible that subjects with lower intake of dietary antioxidants had higher levels of circulating CRP. In support of this notion, in a study on 3,258 apparently healthy British men, plasma ascorbic acid concentration, fruit intake, and dietary vitamin C intake were all inversely associated with CRP and tissue plasminogen activator (t-PA), an endothelial marker

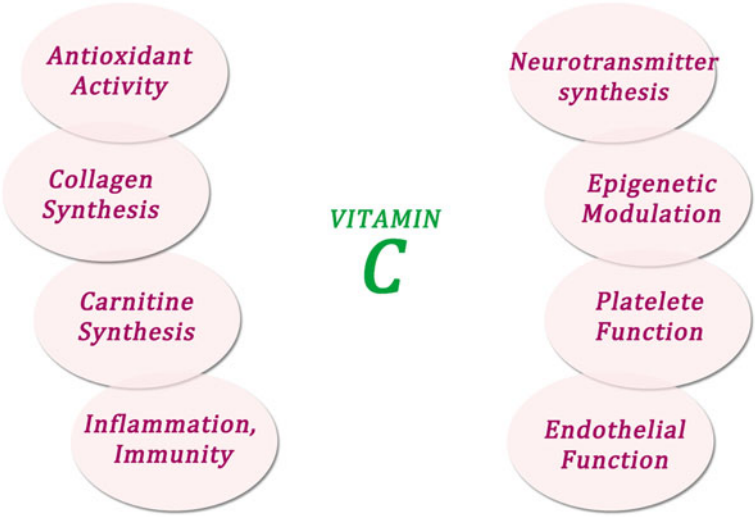

Fig. 6 Vitamin C functions

(Wannamethee et al. 2006). Notwithstanding, a cross-sectional study on 28 hospitalized children with acute lymphoblastic leukemia (ALL), an inflammatory condition with concomitant augmented oxidative stress, and 30 healthy children, presented evidence for inflammation/oxidative stress-induced increased vitamin C utilization. Though vitamin C intake in children with ALL was above twice as much as in controls, their plasma and urinary concentrations of ascorbic acid were less than 10 and 40% of their healthy counterparts. Correspondingly, serum total antioxidant capacity (TAC) in patients was significantly lower than in controls, indicating enhanced vitamin C utilization in ALL patients (Neyestani et al. 2007a).

Effect of Vitamin C on Inflammation

Vitamin C, beyond its main function as a highly effective antioxidant, has a number of activities that could conceivably contribute to its inflammation-modulating effects. An in vitro study reported decreased proinflammatory cytokines TNF-α and IFN-γ and increased anti-inflammatory IL-10 formation by peripheral blood lymphocytes incubated with vitamin C (Molina et al. 2014). The results of a meta-analysis declared that vitamin C supplementation significantly decreases serum CRP concentrations (pooled effect of −0.23 mg/L). A more pronounced effect was observed in participants with a baseline CRP concentration $\geq$ 3 mg/L indicating vitamin C supplementation is more effective for downgrading inflammation in subjects with an activated inflammatory reaction (Jafarnejad et al. 2018).

Because of its modulatory effects on proinflammatory cytokines, vitamin C, like vitamin D, has been considered as a potential therapeutic agent against Covid-19-induced "cytokine storm" and its consequent mortality (Cerullo et al. 2020).

B Vitamins

B vitamins comprised of a chemically diverse nutrients with a wide variety of actions that are sometimes quite inter-related. They include thiamine (B_1), rhiboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine, and related compounds (B_6), biotin (B_7), folic acid (B_9), and cobalamins (B_{12}). Table 2 shows certain characteristics of the B vitamins.

Effect of Inflammation on B Vitamins Status

There is limited evidence for the effect of inflammation on the status of individual B vitamins, and most studies have examined the association between B vitamins and inflammatory biomarkers. For instance, in Atherosclerosis Risk in Communities (ARIC) Study, the association between homocysteine (an intermediary metabolite of methionine whose serum high level is a CVD risk factor), circulating and dietary B vitamins (including B_6, folate, and B_{12}) with multiple inflammatory biomarkers were examined in 519 healthy middle aged adults. However, there was no significant association between endothelial inflammatory biomarkers including intracellular adhesion molecule (ICAM)-1 or vascular cell adhesion molecule (VCAM)-1 with the amounts of B vitamins in diet or blood. Nor was there a relationship between inflammatory markers including CRP with serum homocysteine concentrations (Folsom et al. 2003). Contrary to the findings of this report, in a study conducted on 1976 German women, the associations among plasma vitamin B_6 as pyridoxal-5′-phosphate (PLP), vitamin $B_{12,}$ and erythrocyte folate concentrations with serum CRP, SAA, homocysteine, and cysteine concentrations were investigated. There was an inverse significant correlation between homocysteine and all B vitamin biomarkers. Among the B vitamins, only circulating PLP was inversely correlated with serum CRP and SAA concentrations. In multiple linear regression analysis, plasma PLP, erythrocyte folate, serum homocysteine, and cysteine were found as the independent predictors of CRP (Abbenhardt et al. 2014). However, due to the cross-sectional nature of the study, it is hard to interpret causality of the reported associations between B vitamins status and inflammatory biomarkers. Furthermore, some evidence suggests inflammation-induced compartmentalization of pyridoxal-5′-phosphate (Chiang et al. 2005), and this may also be the case for some other B vitamins. On the whole, the effect of inflammation on individual B vitamins warrants further studies.

Effect of B Vitamins Status on Inflammation

The effect of B vitamins on inflammatory biomarkers has been investigated both in animal models and human subjects. In an experimental study, the association between thiamine with inflammation, oxidative stress, and cellular recruitment in cecal ligation and puncture (CLP)-induced sepsis was investigated in murine model. Mice were fed on either regular or thiamine-deficient chow diet. Finally, in thiamine-deficient CLP mice the peritoneal fluid concentrations of TNF-α and MCP-1 were higher, but serum concentrations of IL-1β were lower than in thiamine sufficient mice. Thiamine may exert its anti-inflammatory effects via downregulation of PPAR-γ (Hu et al. 2018).

Table 2 A summary of B vitamins characteristics (Kohlstadt 2019)

B vitamin	Chemical name	Main functions	Deficiency syndrome	Main dietary sources
B_1	Thiamine	Thiamine pyrophosphate is an essential cofactor for enzymatic decarboxylation of oxoacids	(a) Beriberi, dry and wet types (b) Wernicke Korsakoff syndrome	Whole grains, yeasts, meat, and fish
B_2	Riboflavin	Main component of flavoproteins, FMN and FAD, that are important cofactors of many oxido-reductase enzymes	Stomatitis, cheilosis, sore tongue, ocular symptoms, and anemia	Milk, yogurt, cheese, meat, eggs, chicken, and fish
B_3	Niacin and nicotinamide	A component of the important cofactors, NAD and NADP, that regulate oxido-reduction and phosphorylation reactions	Pellagra syndrome	Fish, beef, chicken, legumes, nuts, and whole grains
B_5	Pantothenic acid	Synthesis of acetyl CoA and acyl carrier protein	Numbness of the hands and feet, extreme tiredness, irritability, sleeping problems, heartburn, diarrhea, nausea, vomiting, and loss of appetite	Beef, chicken, eggs, milk, whole grains, and nuts
B_6	Pyridoxine, pyridoxal, and pyridoxamine	Pyridoxal phosphate is the cofactor of transaminases, involved in decarboxylation reactions	Peripheral neuropathy, sore tongue	Meat, fish, whole grains, and nuts
B_7	Biotin	As a cofactor for carboxylation enzymes	Hair thinning, hair loss, and skin rash	Some fruits, vegetables, whole grains, eggs, meat, and nuts
B_9	Folic acid, folate	Involved in transfer of one-carbon groups mostly as methyl or formyl moieties, DNA synthesis, and repair	Megaloblastic anemia; deficiency state in pregnancy is associated with neural tube defects in newborns	Green vegetables and meats
B_{12}	Cobalamines	Coenzyme in nucleic acid synthesis and conversion of homocysteine to methionine	Pernicious anemia including megaloblastosis plus subacute degeneration of spinal cord	

Riboflavin also has anti-inflammatory activities by suppressing TNF-α, IL-6, MCP-1, and high-mobility group box 1 (HMGB1), an inflammatory nonhistone chromatin-associated protein, and enhancing anti-inflammatory adiponectin and IL-10 (Mazur-Bialy and Pocheć 2016). Riboflavin is a proteasome inhibitor with suppressing effect on NF-κB activation; thereby it may confer protective effect against several inflammatory disorders (Qureshi et al. 2011) and inflammatory nociceptive pain, as well (Bertollo et al. 2006). The anti-inflammatory activity of riboflavin is exerted intracellularly via inhibition of inflammasome, a cytosolic innate immune receptor that regulates the activation of caspase-1, cytokine maturation, and inflammatory lytic form of programmed cell death, i.e., pyroptosis (Ahn and Lee 2020).

The effect of niacin on inflammation has been the subject of several studies. It has been shown that niacin inhibits vascular inflammation through induction of heme oxygenase-1 (Wu et al. 2012), suppresses colonic inflammation and carcinogenesis (Singh et al. 2014), attenuates pulmonary inflammation and improves survival during sepsis by downregulating the NF-κB pathway (Kwon et al. 2011), and ameliorates neuroinflammation in Parkinson's disease via GPR109A, a G-protein-coupled receptor (Giri et al. 2019).

Recent studies have shown the direct interaction of vitamin B_6 with inflammasomes, nuclear receptor corepressors, and coactivators and receptor-interacting protein 140 (a transcriptional coregulator), indicating a role for vitamin B_6 in inflammatory response (Bird 2018). The inverse association between dietary intake of vitamin B_6 and circulating CRP concentrations has been reported (Morris et al. 2010). Along the same line, in a clinical trial on 35 adults with rheumatoid arthritis, 12 weeks supplementation with vitamin B_6 100 mg plus folic acid 5 mg a day ($n_1 = 20$), compared with only folic acid 5 mg/d ($n_2 = 15$), resulted in a significant decrease in IL-6 and TNF-α (Huang et al. 2010).

Some experimental studies suggest an anti-inflammatory function for folate mostly via NF-κB suppression (Ebaid et al. 2013; Feng et al. 2011; Samblas et al. 2018). Evidence from a cohort study indicates an association between both folate and vitamin B_{12} status and inflammatory biomarkers (Guest et al. 2015). However, evidence from human studies investigating the effect of folate supplementation on inflammatory biomarkers is scarce and controversial (Mangoni et al. 2003; Solini et al. 2006).

An inverse association between vitamin B_{12} status and serum concentrations of IL-6 has been reported (Lee et al. 2016). In another study on 364 subjects including children ($n_1 = 224$, 12.99 ± 2.73 y; BMI: 20.07 ± 4.92 kg/m^2) and adults ($n_2 = 140$, 41.87 ± 8.82 y; BMI: 31.65 ± 5.77 kg/m^2), an inverse association between serum concentrations of vitamin B_{12} and TNF-α was found only in adult subjects (Al-Daghri et al. 2016). In a study on hyperhomocysteinemic elderly subjects with the mean age of 72 y from both sexes who were consuming either vitamin B_{12} 500 μg/d and folic acid 400 μg/d ($n_1 = 271$) or placebo ($n_2 = 251$), supplementation for 2 years did not result in any significant change in serum concentrations of endothelial or systemic inflammatory biomarkers including ICAM-1, VCAM-1, and CRP (Van Dijk et al. 2016). However, no data on dietary intake of the participants were presented.

Some studies have investigated the effect of combination of B vitamins on inflammation. In a study on Nepali HIV-positive adult subjects aged 18–60 years (180 males and 134 females), dietary intake of niacin, pyridoxine, or cobalamin was independently and inversely associated with serum CRP concentrations (Poudel-Tandukar and Chandyo 2016). In a human study, the effect of reducing circulating concentrations of homocysteine via long-term supplementation with B vitamins including B_6 (50 mg), folic acid (2.5 mg), and B_{12} (1 mg) on biomarkers of systemic and endothelial inflammation in 300 women at high risk of CVD was examined (150 in treatment and 150 in placebo group). After 7.3 years of intervention, plasma concentration of homocysteine was significantly decreased in the supplemented group (by 18%). However, this was not accompanied by any significant change in inflammatory biomarkers including CRP, IL-6, ICAM-1, or fibrinogen (Christen et al. 2018). One explanation for these discrepancies in findings could be that the anti-inflammatory effects of B vitamins may be more prominent in the context of inflammatory diseases, as compared with low-grade systemic inflammation conditions with much lower concentrations of circulating proinflammatory biomarkers.

Considering Inflammatory Biomarkers in Vitamin Supplementation Studies: When and How

The evidence presented above clearly indicates the effect of inflammation on body status of almost all vitamins. Hence, low vitamin status commonly observed in many chronic diseases may reflect the augmented inflammatory reactions underlying these diseases (Furman et al. 2019). This may, at least in part, explain the discrepancies of the results of different clinical trials. We, therefore, do recommend that in the vitamin supplementation studies aiming at other aspects of chronic diseases than inflammation as the primary outcomes, inflammatory biomarkers like (hs)CRP be considered in the analyses and interpretation of results. Even in population surveys on micronutrient status, though a large sample size can minimize the confounding effect of inflammation that may be present in some participants, determination of an inflammatory biomarker (mostly hs-CRP) and adjustment of vitamin assay results accordingly could be an asset. This consideration is especially necessary when the prevalence of subclinical infection (like Covid-19) or noninfectious inflammation-associated conditions (like overweight/obesity) is remarkable. The fundamental of the methods of adjustment has been proposed (Thurnham and Northrop-Clewes 2016). Briefly, in population studies, four stages of subclinical inflammation may be present: (i) no inflammation (reference group); (ii) recently triggered inflammatory reaction (for instance due to infection) with asymptomatic rising circulating CRP concentration; (iii) early convalescence with diminishing circulating CRP but still disarranged micronutrient status and probably increased α-1-acid glycoprotein concentration; and (iv) late convalescence with elevated serum CRP concentration. Correction factors for vitamin A according to the abovementioned stages of inflammation have been proposed (Thurnham 2015). However, these factors should be calculated for each population and others for other vitamins, as well.

Concluding Remarks for Future Studies

The effects of inflammation on vitamins status still need further elucidation. Along the same line, in 2012 the Center for Disease Control (CDC), National Institute for Child Health and Human Development, and Global Alliance for Improved Nutrition initiated a collaborative research group called Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia (BRINDA). The aim of this study is to make necessary information for the global guidelines on the assessment of micronutrient status (including iron and anemia) and also to develop a research scheme for future prospective studies on micronutrients (Suchdev et al. 2016). Calculation of correction factors for circulating concentration of individual vitamins according to the stage of inflammation and for each population can be the subjects of future studies. Using this approach can help preventing under- or overestimation of the prevalence of vitamin deficiencies in the studied population and explaining the unexpected circulating concentration of the vitamin following supplementation, as well.

In vitamin supplementation studies, especial attention should be paid to the baseline values of both vitamin status and inflammatory biomarkers. Initial high vitamin status and low inflammatory biomarkers concentrations are less likely to be responsive to the intervention. The vitamin supplement dose and frequency of intake are also important factors. In clinical trials with short duration (8–12 weeks), using higher doses a day may be justified.

Finally, we do recommend including dietary assessment in each vitamin supplementation study. The absence of a significant difference of dietary intake in initial and final intra- and inter-group comparisons considerably strengthens the causal relationships between the intervention and outcomes. In the case of changes of dietary intakes, appropriate statistical adjustment methods must be employed.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, the current understanding of the inter-relationships between vitamins and inflammation is reviewed. The overall data shows inverse correlations between inflammatory biomarkers and circulating vitamins (Thurnham and Northrop-Clewes 2016). Though decreased status of some vitamins might be regarded as a predisposing factor for certain diseases (like vitamin D) (Xu et al. 2020), it might also be due to inflammation-induced increased utilization (like vitamin C) (Neyestani et al. 2007a) or, occasionally, sequestration of the vitamins (like vitamin A) (Rubin et al. 2017). These findings altogether indicate that vitamin supplementation may be beneficial for subsiding inflammatory reaction and, finally, the disease outcomes. Though most clinical trials have shown the suppressing effect of vitamin supplementation on inflammatory biomarkers, the overall results of the effect of such interventions on disease outcomes have been disappointing to date (Oliver-Baxter et al. 2018; Rodriguez et al. 2018; Manson et al. 2018). This implies that the relationships among vitamins, inflammation, and disease outcomes are more complicated than what is thought. For better interpretation of vitamin status in the context of inflammation and infection, certain adjustment methods

according to inflammatory biomarkers have been proposed. This approach, also reviewed in this chapter, may help for more appropriate designing of the future supplementation studies in different clinical settings.

Mini Dictionary of Terms

Acute phase proteins or reactants (APPs): A class of circulating proteins whose concentrations either increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in acute phase response.

Acute phase response (APR): The initial response of the organism to infection, inflammation, or trauma in an attempt to return to homeostasis.

Cytokine: Small cell-signaling molecules that function in cell to cell communication in immune response in autocrine, paracrine, and endocrine manners.

Damage-associated molecular patterns (DAMPs): Endogenous molecules that are released from damaged or dying cells and activate innate immune response and interact with pattern recognition receptors (PRRs).

Dendritic cell (DC): A special type of antigen-presenting cell (APC) found in tissues.

Diapedesis: The squeezing of white blood cells in ameboid manner across the intact walls of the capillaries, typically accompanying inflammation.

Freund's adjuvant: An immunoenhancer solution composed of antigen emulsified in mineral oil. Depending on presence or absence of inactivated mycobacteria, there are two forms of complete or incomplete Freund's Adjuvant, respectively.

Hyperalgesia: Extreme sensitivity to feeling pain.

High-sensitivity C-reactive protein: A blood test to measure lower levels of C-reactive protein (CRP).

Hyperhomocysteinemic: A state of high blood homocysteine concentration.

Hypermetabolic state: The condition of increased resting energy expenditure (REE) above 110% of the predicted value.

Hyporetinolemia: Low blood retinol concentrations.

Hypervitaminosis: A condition of abnormally high storage levels of a vitamin that potentially can lead to toxicity.

Immune-modulating property: A property of a substance to either stimulate (immunostimulator) or suppress (immunosuppressor) the immune response.

Immunoenhancer: A substance that potentiates immune response.

Inflammasomes: Cytosolic receptors and sensors of the innate immune system that regulate the activation of caspase-1 and induce inflammation.

Kupffer cells: Macrophages that are residing in the liver.

Leukotrienes: A group of eicosanoid inflammatory mediators produced by leukocytes through oxidation of arachidonic acid by the enzyme 5-lipoxygenase.

Macrophage-chemoattractant protein (MCP)-1: One of the important chemokines that controls migration and infiltration of monocytes/macrophages during inflammation.

Metabolic syndrome (MetS): A cluster of conditions that when occur together, increase the risk of several diseases including cardiovascular disease, stroke, and type 2 diabetes. These conditions include raised blood pressure, high fasting circulating concentrations of glucose and triglycerides, excess body fat around the waist, and low serum concentration of high-density lipoprotein cholesterol (HDL-C).

Noncommunicable disease (NCD): Or chronic disease is a disease that is not transmissible directly from one person to another. They usually have a long duration and result from interaction among environmental (including nutritional), genetic, physiological, and behavioral factors.

Nuclear factor kappa-B (NF-κB): Or nuclear factor kappa-light-chain-enhancer of activated B cells is a transcription factor that organizes cellular resistance to invading pathogens through linking signals from the pathogens and host cells.

Oxidative stress (OS): An imbalance between prooxidants (free radicals) and antioxidants in favor of prooxidants.

Pathogen-associated molecular patterns (PAMPs): Conserved small molecular motifs within a class of microbes that are recognized by toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals.

Pattern recognition receptors (PRRs): Proteins that recognize molecules commonly found in pathogens including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).

Plasminogen activator inhibitor (PAI)-1: A serine protease inhibitor (serpin) that impedes fibrinolysis via inhibition of plasminogen activators, i.e., tissue plasminogen activator (tPA) and urokinase.

Proinflammatory: Capable of causing inflammation.

Prostaglandin: A group of physiologically active lipid-derived compounds called eicosanoids with a wide variety of hormone-like effects in animals.

Provitamin: A substance that can be converted to a vitamin within the body.

Pyroptosis: A form of lytic programmed cell death triggered by proinflammatory signals that is linked to inflammation.

Regulatory T cells (Treg): A specialized subpopulation of T cells that maintain self-tolerance via modulating immune response.

Secosteroid: A subclass of steroids characterized by a cleaved ring.

Tetraterpenoids: Chemically modified tetraterpenes with oxygen-containing functional groups.

T-helper (Th) cell: Also known as $CD4^+$ T lymphocyte is a type of white blood cell that stimulates killer T cells, macrophages, and B lymphocytes (B cells) to mount immune responses.

Total antioxidant capacity (TAC): A measure to evaluate the antioxidant status of biological samples and also the antioxidant response against free radicals.

Vasodilation: Widening of blood vessels due to relaxation of the muscular walls of blood vessels.

Vitamer: Related form of a vitamin that exhibits biological activity against a specific vitamin deficiency.

Key Facts of the Relationship Between Inflammation and Vitamins

- Inflammation is a natural immune response to the foreign invaders to the body.
- Inflammatory reaction must be precisely regulated from initiation to termination to keep the tissues and organs of the body safe.
- Any defect in mounting inflammatory response or in regulating its termination could be in opposition with the survival of the organism.
- Many studies have reported an inverse association between circulating concentrations of vitamins and inflammatory biomarkers.
- Attempts have been made to improve selected vitamin status through supplementation in the course of different diseases to alleviate inflammation.
- Though several clinical trial studies have reported downregulation of inflammatory biomarkers following vitamin supplementation, the overall effect on disease outcomes is still controversial.

Summary Points

- Chronic inflammation-associated disorders are the most prevalent pathologies and the main causes of death in the world.
- Among these disorders, cardiovascular disease (CVD), diabetes, chronic obstructive pulmonary disease (COPD), and autoimmune disorders have the highest occurrence globally.
- Continuous attempts have been made to reinforce the antioxidant defense systems through increased intake of naturally occurring antioxidants in fruits and vegetables or vitamin supplementation to suppress augmented inflammatory response and the resulting oxidative stress.
- Low vitamin status commonly observed in many chronic diseases may reflect the augmented inflammatory reactions underlying these diseases.
- This may, at least in part, explain the discrepancies of the results of different clinical trials.
- We recommend that in the vitamin supplementation studies aiming at other aspects of chronic diseases than inflammation as the primary outcomes, inflammatory biomarkers like (hs)CRP be considered in the analyses and interpretation of results.

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Candidate Biomarkers for Sarcopenia and Relationship with Nutrition

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Abstract

Sarcopenia, originated from the Greek "sarx" (flesh) and "penia" (loss), is mostly a geriatric syndrome, characterized by a progressive decrease of skeletal muscle mass and function. This chapter aims to define a set of biomarkers associated with nutrition that may be used to describe various processes of sarcopenia in different patients, allowing for individualized monitoring and enabling preventative and therapeutic methods. The prevalence of sarcopenia varies according to the characteristics and the living situation of the study population, the diagnostic criteria, and the method used to estimate muscle mass, strength, and physical performance. Several guidelines have been published in order to enhance the early detection, diagnosis, and management of sarcopenia. Furthermore, a variety of biomarkers have been investigated for their role on its early diagnosis. For this reason, a number of biomarkers of nutritional status, were investigated. Vitamin D is a key regulator of bone metabolism because of its role in the regulation of phosphate and calcium homeostasis. Elevated levels of uric acid, with antioxidant properties, may have a protective role against excessive radical-free species. Lower serum n-3 levels were significantly associated with a higher risk of sarcopenia; thus, n-3 FAs could have a protective effect on human muscle homeostasis. Adipose tissue secretes leptin and proinflammatory cytokines, which stimulate muscle catabolism, thus triggering a vicious cycle that leads more rapidly to physical disability and sarcopenia. Sarcopenia is associated with increased levels of CRP, IL-6, and sIL-6r. Serum proteins, such as: a) prealbumin, b) albumin, c) transferrin, and d) retinol-binding protein (RBP), can be used to detect malnutrition in the elderly. Creatinine is a reliable biomarker for muscle mass because of its easy accessibility and cost-effectiveness. Low concentration of IGF-1 is associated with skeletal muscle mass loss, which probably plays a crucial role in the development of sarcopenia. Therefore, there is increasing interest in dietary antioxidants and their effects on age-related losses of muscle mass and function. Due to the multifactorial genesis of sarcopenia, it is crucial to identify different biomarkers. In conclusion, nutrition is associated with muscle mass, strength, and function in older adults and has an important role in the prevention and management of sarcopenia.

Keywords

Sarcopenia · Biomarkers · Nutrition · Vitamin D · Leptin · Insulin-Growth Factor 1 · Visceral Proteins

Abbreviations

BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
CAF	C-terminal agrin fragment
CRP	C-reactive Protein
DXA	Dual-Energy X-ray Absorptiometry
EWGSOP	European Working Group on Sarcopenia in Older People
GDF-15	Growth-Differentiation Factor-15
IGF-1	Insulin-Like Growth Factor 1
IL-8	Interleukin 8
LBM	Lean Body Mass
MPO	Myeloperoxidase
mTOR	Mechanistic target of rapamycin
PDGF-BB	Platelet-Derived Growth Factor-bb
PUFA	Polyunsaturated Fatty Acid
sIL-6r	Soluble Interleukin 6 Receptor alpha

Introduction

Sarcopenia, originated from the Greek "sarx" (flesh) and "penia" (loss), is mostly a geriatric syndrome, characterized by a progressive decrease of skeletal muscle mass and function (Rosenberg 1997). It is one of the most common health problems among the elderly, and it raises the risk of frailty, falls, and injuries leading to hospitalization, loss of independence, morbidity, and mortality (Senior et al. 2015).

It is important to understand the risk factors that contribute to the development of sarcopenia in order to successfully prevent this condition. Age-related changes in muscle fiber structure are observed, such as modifications of its contractile properties and abnormalities of neuromuscular junctions (Casati et al. 2019). In sarcopenia, there is a predominance of type I fibers and an atrophy of type II fibers causing a decrease in both muscle strength and physical function (Larsson et al. 1979), as well as an accumulation of adipose tissue both around and between muscle fibers (Goodpaster et al. 2001). In addition, the phenotype of sarcopenia in aging can be caused and/or exacerbated by various comorbidities. Among the risk factors of sarcopenia are third age (Chen et al. 2021; Santilli 2014), low level of physical activity, malnutrition (Papadopoulou 2020), body mass index (Chen et al. 2021), and several comorbidities, as cardiovascular diseases, diabetes, respiratory diseases (Pacifico et al. 2020), human immunodeficiency virus (HIV) (Bonato et al. 2020), and cancer (Marhold et al. 2021). Nevertheless, despite the significant overlap in the severity of sarcopenia phenotype and comorbidities, sarcopenia is commonly underdiagnosed in clinical practice (Kwak et al. 2018).

Furthermore, sarcopenia has become more complicated involving multiple pathogenesis mechanisms, including endocrine dysfunction, growth factors, neuromuscular junction, muscle protein changes, and inflammatory conditions (Curcio et al. 2016). As a result, in recent years several circulating variables have been considered as potential

biomarkers (Picca et al. 2021) for measuring sarcopenia. Specific biomarkers linked to clinical assessments have been proposed, allowing the detection of older adults likely to suffer from sarcopenia and monitoring, also, the effectiveness of prevention and treatment strategies. This chapter aims to define a set of biomarkers associated with nutrition that may be used to describe various processes of sarcopenia in different patients, allowing for individualized monitoring of the success of preventative and therapeutic methods.

Criteria Defining Sarcopenia

Several guidelines have been published in order to enhance the early detection, diagnosis, and management of sarcopenia. In 2010, the European Working Group on Sarcopenia in Older People (EWGSOP) recommended cutoff values for muscular mass, strength, and physical performance as a practical clinical definition of sarcopenia (Cruz-Jentoft et al. 2010). A consensus, similar to the EWGSOP, has been published in 2011 by the International Working Group on Sarcopenia (IWGS) (Fielding et al. 2011). In 2014, the Foundation for the National Institute of Health (FNIH) Sarcopenia Project published their consensus (Studenski et al. 2014). Also, the same year, the Asian Working Group for Sarcopenia (AWGS), based on epidemiological data collected from Japanese and other East Asian populations, published guidelines for the diagnosis of sarcopenia (Chen et al. 2014a). Another definition of sarcopenia was published in 2020, by Sarcopenia Definition and Outcomes Consortium (SDOC). According to this scientific group, muscle strength and physical performance defined sarcopenia (Bhasin et al. 2020). The criteria used by each scientific group for sarcopenia are shown in Fig. 1.

It is notable that the EWGSOP updated their definition of sarcopenia, in 2018, in order to incorporate scientific data that had been accumulated over the last decade. So, sarcopenia is detected when low muscular strength coexists with low muscle quantity and quality. Severe sarcopenia is identified when both low muscular strength and low muscle quantity and quality coexist with poor physical performance. Furthermore, EWGSOP 2 recommended simple and clear cutoff points for assessment of factors that identify and characterize sarcopenia (Cruz-Jentoft et al. 2019).

Measuring Methods of Sarcopenia

Magnetic resonance imaging (MRI), computed tomography (CT), dual energy X-ray absorptiometry scans (DXA), and bioelectrical impedance analysis (BIA) are among the technologies used to assess muscle mass (Kwak et al. 2018). Nevertheless, all of them have significant pitfalls. CT and MRI have drawbacks of technological complexity, high costs, and space requirements. DXA measurements can be influenced by body thickness, hydration status, and several diseases associated with body water retention (Tosato et al. 2017). DXA is, also, unable to detect intramuscular adipose tissue, making muscle quality evaluation difficult (Tosato et al. 2017). BIA is less sensitive and specific in comparison to the other methods, since the density of skeletal

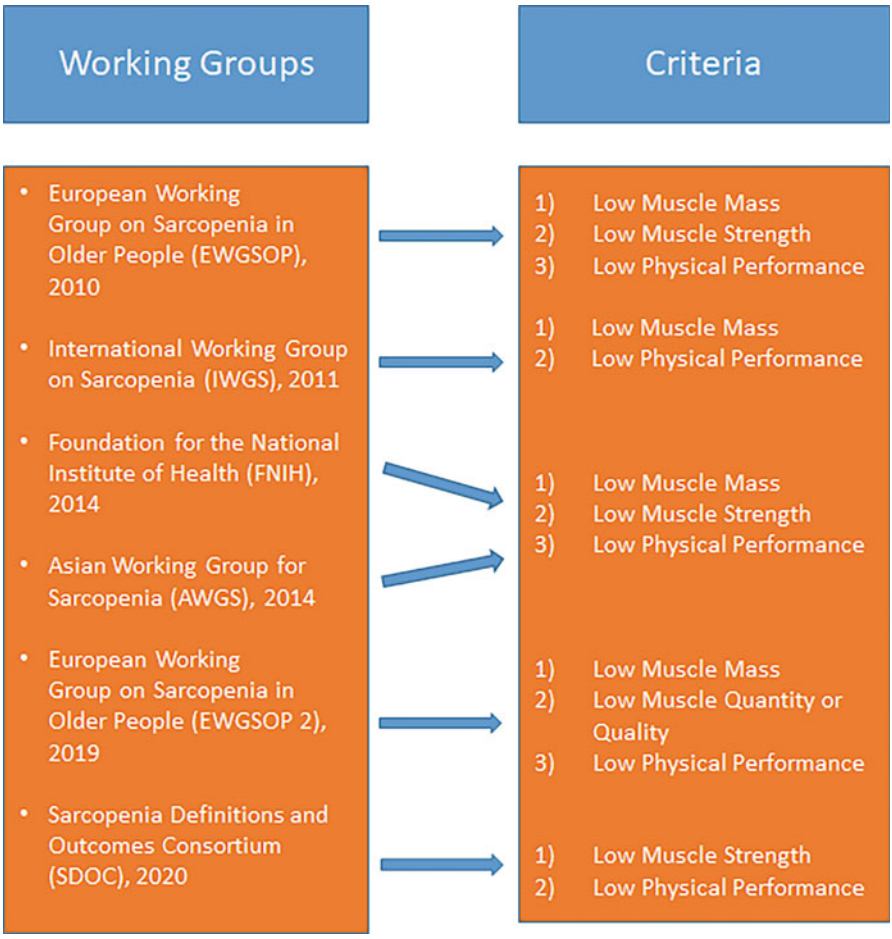

Fig. 1 The working groups and their recommended criteria for sarcopenia

muscle cannot be determined (Aleixo et al. 2020) and measurements can be potentially affected by the hydration condition of the patient (Cruz-Jentoft et al. 2019). Given these limitations, DXA is still considered the current reference technique for the assessment of muscle mass in clinical practice (Beaudart et al. 2016).

Muscle strength is measured by handgrip strength using a dynamometer, an inexpensive and simple method (Cruz-Jentoft et al. 2019) which is the recommended approach for assessing muscle strength (Roberts et al. 2011). Leg extension strength consists, also, another method to measure the muscular strength in the lower body (Martone et al. 2019). On the other hand, physical performance can be assessed by the Short Physical Performance Battery (SPPB) test, gait speed test, and the Timed-Up and Go test (TUG) (Cruz-Jentoft et al. 2019; Martone et al. 2019). The most widely accepted tools for measuring muscle mass, muscle strength, and physical performance as well as serum biomarkers for detection and diagnosis sarcopenia are described in Fig. 2.

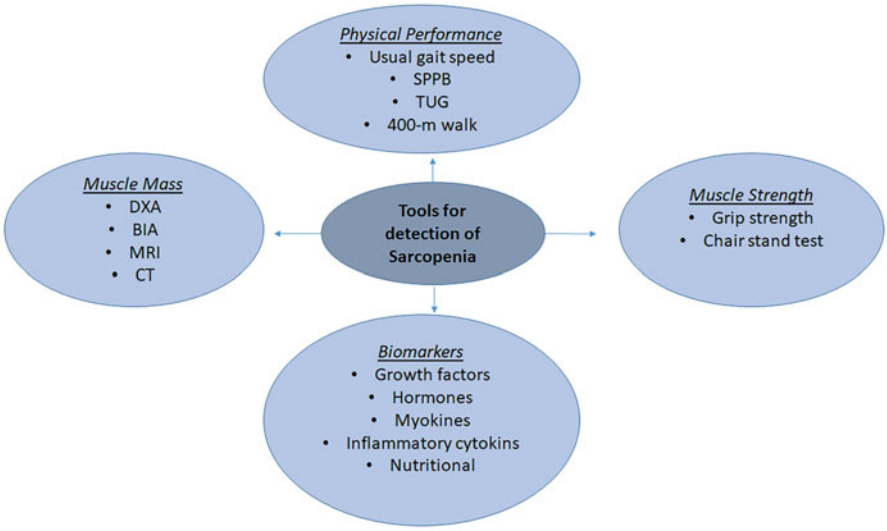

Fig. 2 Tools for measurement of muscle mass, muscle strength, and physical performance and biomarkers for sarcopenia detection and diagnosis. DXA = Dual energy X-ray absorptiometry; BIA = Analysis or Bioimpedance Analysis; MRI = Magnetic Resonance Imaging; CT = Computed Tomography; SPPB = Short Physical Performance Battery; TUG = Time Up and Go; 400-meter walk

Prevalence of Sarcopenia

The prevalence of sarcopenia varies according to a) the characteristics (race, ethnic differences in body composition) and the living situation of the study population (hospitalized, community-dwelling, and nursing home residents), b) the diagnostic criteria, and c) the methods used to estimate muscle mass, strength, and physical performance. In 2019, a systematic review and meta-analysis, based on data from 41 studies and a total of 34,955 participants, found that the prevalence of sarcopenia in community-dwelling adults was 9% and 11% in women and men. The respective values in nursing homes residents were 51% in men and 31% in women, while in hospitalized individuals were 23% for men and 24% for women (Papadopoulou 2020). Furthermore, higher prevalence of sarcopenia was detected between non-Asian countries than Asian countries, especially when the BIA method was used, in both genders (19% and 20% vs. 10% and 11% for men and women, respectively). However, measurements of DXA method showed higher prevalence among Asian than non-Asian men (9% and 6%, respectively), but in women the prevalence was higher in non-Asian countries compared to Asian (10% and 6%, respectively) (Shafiee et al. 2017).

Biomarkers of Sarcopenia

A plethora of biomarkers have been investigated for their role in early diagnosis of sarcopenia. For this purpose, several studies have selected potential candidate biomarkers and introduced experimental analysis for the confirmation of their importance in the progression of the disease. The candidate biomarkers and their association with sarcopenia and other diseases are shown in Tables 1, 2, and 3.

Enzyme-linked immunosorbent assay (ELISA) technique was used in order to measure the concentration levels of 21 potential serum biomarkers between healthy and sarcopenic elderly people. These biomarkers including proteins related to

Table 1 Biomarkers with increased levels in sarcopenia

Biomarker	Mechanism	Pathogenesis	Associated disease	Reference
C-terminal agrin fragment	Neuromuscular junctions	Impairment of neuromuscular junctions	Immobilization syndrome, chronic kidney disease	Francesco Curcio et al. (2016)
Growth differentiation factor-15	Growth factors	Muscle growth suppressor	Cancer, cachexia, and muscular dystrophy	Francesco Curcio et al. (2016)
Transforming growth factor β	Growth factors	Muscle growth suppressor	Cancer, cachexia, and muscular dystrophy	Ju Yeon Kwak et al. (2018)
Interleukin 6	Inflammation factors	Inflammation	Chronic degenerative disease	Ju Yeon Kwak et al. (2018)
Secreted protein acidic and rich in cysteine	Tissue remodeling	Inflammation	Cachexia, muscular dystrophy	Ju Yeon Kwak et al. (2018)
Macrophage migration-inhibitory factor	Inflammation factors	Inflammation	Cancer, cachexia, and muscular dystrophy	Ju Yeon Kwak et al. (2018)
C-reactive protein	Inflammation factors	Inflammation	Muscular dystrophy	Nafiseh Shokri-Mashhadi et al. (2021)
High-sensitivity C-reactive protein	Inflammation factors	Inflammation	Muscular dystrophy	Nafiseh Shokri-Mashhadi et al. (2021)
P-selectin	Inflammation factors	Inflammation	Chronic degenerative disease	Riccardo Calvani et al. (2021)
IFN-γ-induced protein 10	Inflammation factors	Inflammation	Chronic degenerative disease	Riccardo Calvani et al. (2021)

Table 2 Biomarkers with reduced levels in sarcopenia

Biomarker	Mechanism	Pathogenesis	Associated disease	Reference
Irisin	Growth factors	Muscle growth promoter	Cancer, cachexia, and muscular dystrophy	Francesco Curcio et al. (2016)
Insulin-like growth factor 1	Somatopause	Muscle growth promoter	Cancer, cachexia, and muscular dystrophy	Francesco Curcio et al. (2016)
Tumor necrosis factor α	Inflammation factors	Inflammation	Chronic degenerative disease	Francesco Curcio et al. (2016)
Interleukin 1	Inflammation factors	Inflammation	Chronic degenerative disease	Francesco Curcio et al. (2016)
Macrophage inflammatory protein 1β	Inflammation factors	Inflammation	Chronic degenerative disease	Riccardo Calvani et al. (2020)
Myeloperoxidase	Immune system	Inflammation	Immune deficiency	Riccardo Calvani et al. (2020)
Interleukin 8	Inflammation factors	Inflammation	Muscular dystrophy	Riccardo Calvani et al. (2020)
Macrophage inflammatory protein 1α	Inflammation factors	Inflammation	Muscular dystrophy	Riccardo Calvani et al. (2020)
Platelet-derived growth factor BB	Growth factors	Cell growth	Cancer, cachexia, and muscular dystrophy	Riccardo Calvani et al. (2020)
Alpha-aminobutyric acid	Glutathione synthesis regulation	Impaired catabolism methionine, threonine, and serine	Impaired protein metabolism	Riccardo Calvani et al. (2020)
Asparagine, aspartic acid, and citrulline	Muscle-specific and interorgan processes, the metabolism of proteins	Impaired homeostasis of nitrogen and glutamine	Impaired protein metabolism	Riccardo Calvani et al. (2020)

(continued)

Table 2 (continued)

Biomarker	Mechanism	Pathogenesis	Associated disease	Reference
Phosphoethanolamine	Intermediate of the CDP ethanolamine pathway	Impaired glycerophospholipid synthesis	Impaired synthesis of biological membrane structural functions	Riccardo Calvani et al. (2020)
Catalase	Antioxidant factors	Inflammation	Chronic degenerative disease	Carmen Sánchez-Castellano et al. (2020)

Table 3 Inconclusive biomarkers

Biomarker	Mechanism	Pathogenesis	Associated disease	Reference
Butyryl-cholinesterase	Inflammation factors	Inflammation	Chronic degenerative disease	Francesco Curcio et al. (2016)
Oxidized low-density lipoprotein	Prooxidant	Muscle growth promoter	Chronic degenerative disease	Francesco Curcio et al. (2016)
Testosterone/ dehydroepiandrosterone	Growth factors	Growth promoter	Andropause	Francesco Curcio et al. (2016)
Skeletal muscle-specific troponin T/ N-terminal type III procollagene	Muscle protein turnover	Muscle impairment	Muscular dystrophy	Francesco Curcio et al. (2016)
Monocyte chemoattractant protein 1	Immune system	Inflammation	Immune deficiency	Anna Picca et al. (2020)
Ethanolamine, taurine, threonine, glutamic acid, and sarcosine	Muscle protein turnover	Immobilization syndrome	Muscle dystrophy	Anna Picca et al. (2020)
Myostatin/ activins A and B/ follistatin/ bone-morphogenetic proteins/ brain-derived neurotrophic factor/ 3-methylhistidine	Growth factors	Muscle growth suppression/ promotion	Cancer, cachexia, and muscular dystrophy	Francesco Curcio et al. (2016)
Creatinine	Muscle protein turnover	Muscle impairment	Muscular dystrophy	Francesco Curcio et al. (2016)

skeletal muscle function and metabolism were the following: muscle-derived cytokines (myokines), angiotensin-converting enzyme (ACE), insulin-like growth factor 1 (IGF-1), procollagen-type III N-terminal peptide (P3NP), fibroblast growth factor 21 (FGF21), myostatin, growth differentiation factor 11 (GDF11), meteorin-like (METRNL), macrophage migration-inhibitory factor (MIF), fatty acid-binding protein 3 (FABP3), ciliary neurotrophic factor (CNTF), secreted protein acidic and rich in cysteine (SPARC), brain-derived neurotrophic factor (BDNF), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 15 (IL-15), interleukin 1β (IL-1β), transforming growth factor β 1 (TGFβ1), monocyte chemotactic protein 1 (MCP-1), and vascular endothelial growth factor (VEGF). The candidate biomarkers that seem promising, are those that are significantly different between the sarcopenic group of people and the control group were the following: *IL-6, SPARC, MIF, and IGF-1*. Serum levels of these four biomarkers were increased in sarcopenic elderly people. Given the low levels of sensitivity of this specific method of analysis, further investigation of this findings would be important, for the description of not only qualitative but also quantitative conclusive results that would suggest that this group of biomarkers can be used to predict and describe sarcopenia (Kwak et al. 2018).

Inflammatory Biomarkers

The association between chronic inflammation and sarcopenia onset and progression in elderly people has been of interest for a long time. As part of a meta-analysis study, involving all relevant observational studies, *CRP (C-reactive protein)* and *hs-CRP (high-sensitivity C-reactive protein)* serum concentration levels were associated with sarcopenia. Specifically, there are three diagnostic criteria of sarcopenia: inflammatory markers and disability, impaired musculoskeletal functioning, and muscle strength. Those were attempted to be linked with specific biomarkers which could be useful as predictive indices for early diagnosis. Of those three, muscle strength decrease was successfully linked with the increase of these specific inflammatory biomarkers. Further investigation is necessary, in order to exclude correlation (Shokri-Mashhadi et al. 2021).

In different studies, there has been an attempt to associate biomarkers relevant to progression of immunosenescence, to decrease in micronutrient intake and to insufficient muscle regeneration due to oxidative stress. The following biomarkers have been included: elevated concentration of C-reactive protein (CRP) and insufficient concentrations of myeloperoxidase (MPO), Heat shock protein 72 (HSP72), interleukin (IL) 1, 6, and 8, macrophage-inflammatory protein 1 (MIP1), monocyte-chemoattractant protein 1, and platelet-derived growth factor-BB (PDGF-BB). Regarding the metabolic amino acid markers, that could be useful in a multicomponent approach of sarcopenia prediction, asparagine, aspartic acid, citrulline, ethanolamine, glutamic acid, sarcosine, taurine, threonine should be included in the list of candidate biomarkers (Picca et al. 2020).

Other studies have, also, attempted to narrow down the potential biomarkers for sarcopenia. One important addition, to the list of candidate biomarkers, is MIP-1β

(macrophage-inflammatory protein 1β). Lower serum levels of this molecule could, also, be useful in the discrimination between sarcopenic patients and healthy population, as part of a multifaceted control, along with MPO, IL8, MIP-1α (Macrophage inflammatory protein 1α), and PDGF-BB, as mentioned above. On the other hand, the higher serum levels of P-selectin, CRP, and IFN-γ-induced protein 10 (IP-10) could be used as an indication (Calvani et al. 2021).

Biomarkers Related to Protein Metabolism

A number of amino acids could have an indicative role as a fingerprint of the onset of the impairment of the mechanisms associated with sarcopenia. Of course, taking into consideration the correlation between all these mechanisms, a specificity in every amino acid's contribution as a biomarker is required. Insufficient levels of alpha-aminobutyric acid could be related to an impaired catabolism of methionine, threonine, and serine leading to an insufficient regulation glutathione synthesis. To this specific metabolic pathway are attributed conditions of oxidative stress. A great predictive role could be also attributed to the collective concentration levels of asparagine, aspartic acid, and citrulline because of their important contribution to muscle-specific and interorgan processes, the metabolism of proteins, and general homeostasis of nitrogen and glutamine. Similarly, low levels of phosphoethanolamine, as an intermediate of the CDP ethanolamine pathway, are crucial for glycerophospholipid synthesis, and the biological membrane-structural functions should be monitored as an indication of sarcopenia (Calvani et al. 2021).

Neuromuscular Biomarkers

Several studies have dedicated its purpose, in describing the mechanisms contributing to the progression of sarcopenia and therefore predicting its onset using all the related biomarkers. First of all, the gradual loss of neurophysiological functions could describe the pathophysiological condition of a sarcopenic patient and is associated with dysfunction of neuromuscular junctions (NMJs). Agrin is a protein which most likely takes part in the activation of tyrosine kinase muscle-specific receptor (MuSK), stabilizing the acetylcholine receptor (AChR). A C-terminal agrin fragment is derived through the proteolytic cleavage, a synaptic origin protease. This biomarker seems to impair the AChR function, leading to diminishing neurophysiological condition. Several studies have indicated elevated circulating levels of C-terminal argin fragment (CAF) in sarcopenic patients as opposed to healthy population (Curcio et al. 2016).

Hormonal Biomarkers

Insufficient levels for a number of hormones, such as sex hormones (e.g., testosterone that increases muscle protein synthesis and dehydroepiandrosterone (DHEA)), growth hormones, and insulin-like growth factor 1 (IGF-1) could also be of

importance in the prediction and characterization of sarcopenia. One related growth factor with a potential role as biomarker is the Growth Differentiation Factor-15 (GDF-15). More specifically, protein and mRNA levels of GDF-15 could show significant correlation with muscle impairment. A key role has been attributed to Irisin (IR), in its contribution to bone, fat, and muscle metabolism. Preliminary data has shown that its negative relationship with sclerostin, a protein with a key role in bone structural factors, could suggest its value as a biomarker in sarcopenia (Curcio et al. 2016). From a different point of view that implicates the inflammatory mechanisms inducing sarcopenia yet another time, a number of studies have concluded to the importance of proinflammatory cytokines and specifically to those of interleukins (IL-6, IL-1) and tumor necrosis factor alpha (TNF-alpha). These cytokines, produced in the adipose tissue, have been found in increased levels in sarcopenic patients (Curcio et al. 2016). In a large study conducted in very old patients with hip fracture with and without sarcopenia, a wide range of molecules was assessed for their potential role as a biomarker in sarcopenia. This study showed that only TNF-α (lower peripheral tumor necrosis factor-α) and CAT (catalase) levels differed between healthy and sarcopenic patients with hip fracture. For the rest of the neuromuscular, proinflammatory, or oxidative stress markers studied, no significant difference was found (Sánchez-Castellano et al. 2020).

Biomarkers Associated to Malnutrition

The behavioral factors, which are significant in the development of sarcopenia, include the following: a) nutritional status, b) obesity status, and c) physical activity level (Papadopoulou 2020). Specifically, malnutrition is very important in the development of sarcopenia. As a result, a plethora of biomarkers are suggested to assist the diagnosis of sarcopenia. The remainder of this chapter contains biomarkers of nutritional status, as that could be used to detect sarcopenia. The biomarkers associated with sarcopenia and related to diet are listed below and presented in Table 4.

Vitamin D

Vitamin D is a key regulator of bone metabolism because of its role in the regulation of phosphate and calcium homeostasis, and it is strongly associated with muscle strength (Houston et al. 2007; Ward et al. 2009) and physical performance (Houston et al. 2007). Vitamin D affects the number and diameter of type II muscle cells, especially type IIA, which induce rapid muscle contraction velocity and are vital for a short duration, high-intensity anaerobic activities such as acceleration and deceleration, sprinting, and jumping (Agergaard et al. 2015). Vitamin D status acts as a useful biomarker for predicting total mortality, hip fractures, early death, and the development of sarcopenia (Caristia et al. 2019). Nevertheless, further research is needed to fully describe the underlying mechanisms of action of vitamin D in human muscle tissue.

Table 4 Nutritional biomarkers for sarcopenia

Biomarker	Notes
Vitamin D	A useful biomarker for predicting the development of sarcopenia
Uric acid	Higher levels associated with: a) higher handgrip strength and b) better muscle function
n-3 fatty acid	Lower serum n-3 levels are associated with a higher risk of sarcopenia, mainly due to their anti-inflammatory capacity
Prealbumin	Although it is a sensitive indicator of nutrient deficiency, it should be used as part of a comprehensive assessment program
Albumin	Low serum albumin also used for decades as an index of malnutrition
Transferrin	Serum levels are reduced in severe malnutrition, but this marker was found to be unreliable in the assessment of mild malnutrition and lean mass in elders
RBP	RBP measurement is not widely applied
Creatinine	Is a reliable muscle mass biomarker, which is a) inexpensive, b) accessible, and c) useful for assessment of skeletal muscle mass
Insulin-like growth factor (IGF-1)	It is an index of malnutrition and could play a crucial role in maintaining skeletal muscle mass quality
Selenium	These antioxidants seem to be inversely correlated with the determinants of sarcopenia
Carotenoid	
Vitamin C + E	

Uric Acid

Oxidative stress has a crucial role in muscle loss caused by physical inactivity and sarcopenia in elders (Meng and Yu 2010). Thus, elevated levels of uric acid, which has antioxidant properties, may have an important role in protection against excessive production of free radical (Kawamoto et al. 2016). Higher levels of serum uric acid are associated with higher handgrip strength (Lee et al. 2019; Macchi et al. 2008; Nahas et al. 2021) and with better muscle function in elders and thus may slow the progression of sarcopenia.

N-3 Fatty Acids

Overall, n-3 fatty acids have a protective effect against chronic metabolic diseases through their anti-inflammatory capacity (Lorente-Cebrián et al. 2013). It is known that chronic inflammation resulting from immune dysfunction and increase in age-related proinflammatory cytokines (such as TNF-α and IL-6) contributes to muscle wasting (Merritt et al. 2013). Therefore, the reduction of inflammation by n-3 FAs is a very likely mechanism explaining their beneficial effects against sarcopenia (Jang et al. 2020). In addition, n-3 FAs can activate mTOR signals. The mTOR pathway is a major anabolic factor for skeletal muscle formation and muscle protein synthesis (Dupont et al. 2019). As a result, n-3 FAs through stimulating the Mtor-signaling pathway could help in the treatment of age-related anabolic

resistance via increasing muscle protein synthesis rate (Dupont et al. 2019). In conclusion, lower serum n-3 levels were significantly associated with a higher risk of sarcopenia, while n-3 FAs could act protectively on human muscle homeostasis (Jang et al. 2020).

Leptin

Leptin is secreted by adipose tissue and is one of the most well-known adipokines. It is a 16-kDa protein that was identified as a product of the obesity gene and plays an essential role in the control of appetite, insulin sensitivity, inflammation, and fat deposition (Kwon and Pessin 2013). Further increases in leptin, mainly due to age-related increases in fat mass, may lead to leptin resistance and thus to a decrease in fatty acid oxidation in muscle. This can lead to ectopic fat deposition in organs, such as the heart, liver, and muscles, and consequently reduce muscle quality in obese older people (Unger 2005). Furthermore, leptin promotes the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-12 (IL-12) (Tilg and Moschen 2006). These cytokines have been proposed to be associated with sarcopenia. Sarcopenia and obesity share common pathophysiological mechanisms, which include proinflammatory cytokines, insulin resistance, oxidative stress, hormonal changes, and functional impairment (Choi 2016). The inflammatory state to which cytokines and adipokines contribute plays a key role in the development of sarcopenic obesity (Schrager et al. 2007). On the other hand, as mentioned before, adipose tissue secretes leptin and proinflammatory cytokines, which stimulate muscle catabolism (Roubenoff et al. 1997), thus triggering a vicious cycle that leads more rapidly to sarcopenia and, eventually, to physical disability (Schrager et al. 2007).

As mentioned already, serum leptin has associated with sarcopenic obesity (Yang and Chen 2021). Sarcopenic obesity is, also, associated with increased levels of CRP, IL-6, and sIL-6r. IL-6 may have both inflammatory and anti-inflammatory properties. Fat mass distribution is, also, important because central obesity tends to be more proinflammatory than general obesity (Schrager et al. 2007).

Visceral Proteins

Serum proteins, such as: a) prealbumin, b) albumin, c) transferrin, and d) retinol-binding protein (RBP), can be used to detect malnutrition in the elderly.

Prealbumin (or transthyretin, TTR) is a thyroid hormone transport protein that is produced by the liver and partially catabolized by the kidneys. Malnutrition is linked to serum prealbumin levels less than 10 mg/dL (Beck and Rosenthal 2002). The use of prealbumin as a nutritional marker has been promoted, particularly during refeeding in the elderly. The TTR parameter has positive associations with muscle mass loss in the elders. TTR production in the liver is reduced in both cytokine-induced inflammatory disorders and protein-deficient conditions. As a result,

decreased TTR values can be used to assess the degree of involutional LBM processes by stratifying the hepatic and muscle-functional abnormalities generated by protein restriction (Ingenbleek 2019). Although the prealbumin level is a sensitive indicator of nutrient deficiency, it should be used as a part of a comprehensive assessment plan (Beck and Rosenthal 2002).

Low serum level of *albumin* is associated with low muscle mass and function (Cabrerizo et al. 2015; Snyder et al. 2012; Visser et al. 2005). Low-serum albumin value has, also, been used for decades as an index of malnutrition. Low-serum albumin level, called hypoalbuminemia, is a predictor of mortality in the elderly whether they are community-dwelling, nursing residents, or hospitalized. The inflammatory condition and, particularly, high concentrations of IL-6 and TNF-α are two of the main factors influencing hypoalbuminemia (Cabrerizo et al. 2015). Serum albumin concentrations are reduced: a) during reduced synthesis due to inflammatory cytokines or liver failure, b) in nephrotic syndrome, and c) in enteropathies and gastrointestinal disorders (Takeda et al. 2003).

On the other hand, serum albumin and prealbumin levels in otherwise healthy individuals with severe nutrient deprivation due to poor access to food or unwillingness to eat, mainly due to anorexia nervosa, were maintained even in the presence of significant weight loss and decreased only during extreme malnutrition (BMI $<$ 11 kg/m2) (Lee et al. 2015). Therefore, the data on these visceral serum proteins and their ability to predict nutritional deprivation is not entirely clear.

Transferrin has also been used as an index of nutritional status (Shetty et al. 1979). Some authors found transferrin measurements helpful for assessing nutritional status (Fletcher et al. 1987), while others did not (Roza et al. 1984). Serum transferrin levels are reduced in severe malnutrition, but this marker was found to be unreliable in the assessment of mild malnutrition and lean mass in elderly patients (Sergi et al. 2006).

Retinol-Binding Protein (RBP) is a required protein for the transport of retinol from the liver to target organs (Bellovino et al. 2003). Retinol deficiency may lead to a reduction in serum levels of RBP because of a decrease in its hepatic synthesis (Smith et al. 1998). However, nephropathic patients suffering from protein-calorie malnutrition may have normal serum RBP levels (Kergoat et al. 1987). On the other hand, mean values of prealbumin and RBP were significantly decreased in underweight subjects and had a higher correlation with FFM compared to albumin (Shenkin et al. 1996). In comparison to prealbumin, RBP is more difficult to measure, and as a result, RBP measurement is not widely applied (Keller 2019).

Creatinine

Creatinine is the end product of creatine, a three-amino-acid compound mainly found in muscle (Keller 2019). Creatinine excretion rate (CER) is considered a useful indirect method for assessing body composition in healthy adult subjects (Virgili et al. 1994). In healthy individuals, creatinine excretion has normal range

from 18 to 21 mg/kg Cr in women and from 21 to 25 mg/kg Cr in men (Heymsfield et al. 2014). Each mmol of creatinine in urine is produced by 1.9 kg of skeletal muscle (Shenkin et al. 1996). Urinary creatinine concentrations are significantly reduced in people with protein balance (Hart et al. 2000). The serum creatinine level should be measured frequently, as it is an indicator of the condition of the skeletal muscles (Curcio et al. 2016). The disadvantages of creatinine are that it is dependent on the nephrological function and its measurement requires urine collection for 24 hours (Keller 2019). However, creatinine is a reliable biomarker for muscle mass status because of its easy accessibility and cost-effectiveness (Patel et al. 2013).

Insulin-Like Growth Factor (IGF-1)

IGF-1 is mainly produced by the liver (Ohlsson et al. 2009). Fasting lowers plasma IGF-1 levels, and its concentrations are stimulated by food intake (Ohlsson et al. 2009). A link has been reported between energy intake (and to a lesser extent protein intake) and plasma IGF-1 levels (Isley et al. 1983). IGF-1 concentrations are altered by renal dysfunction, liver disease, and severe trauma (Shenkin et al. 1996). In addition, insulin-like growth factor-1 (IGF-1) has been involved in several anabolic pathways in skeletal muscle. A low concentration of IGF-1 is associated with skeletal muscle mass loss, which probably plays a crucial role in the development of sarcopenia (Bian et al. 2020). A disadvantage of IGF-1 measurements is that their serum concentrations are affected by other factors, such as the acute phase response (Keller 2019).

Antioxidants

Oxidative stress has been associated with muscle strength decrease and sarcopenia during aging. Reactive oxygen species (ROS) can cause damage to proteins, fats, and DNA. The actions of ROS are usually countered by antioxidant defense mechanisms, such as glutathione peroxidase and thioredoxin, as well as by exogenous antioxidants obtained from the diet, such as carotenoids, selenium, flavonoids, tocopherols, and other plant polyphenols (Semba et al. 2007). Therefore, there is increasing interest in the role of dietary antioxidants and their effects on age-related losses of muscle mass and function.

Low selenium status has been, also, associated with low muscle mass in the elderly (Chen et al. 2014b). Additionally, higher plasma carotenoid levels were associated with a) a higher risk of increased IL-6 level (Semba et al. 2007) and b) a lower risk of severe disability in walking among elders (Lauretani et al. 2008). Furthermore, vitamin C and E attenuate some muscle adaptations to strengthen training in older men (Bjørnsen et al. 2016). Further evidence on the effect of antioxidants on muscle mass and function is needed.

Conclusion

Sarcopenia is a multifactorial syndrome, which has several risk factors that are involved in its development, such as physical inactivity, malnutrition, oxidative stress, comorbidities, and inflammation. Even though sarcopenia and aging share common molecular and cellular mechanisms, there is still no definitive pathophysiological field for these conditions. Due to the multifactorial genesis of sarcopenia, it is crucial to emphasize the importance of identifying different biomarkers.

A proper combination of balance diet and physical activity, both aerobic and nonaerobic, is associated with maintaining of muscle mass, strength, and function in older adults and acts protectively against. The prevalence of malnutrition observed in older populations underlines the need to ensure that all older people are effectively supported to have adequate dietary intake and satisfactory nutritional status. Routine screening and early diagnosis of malnutrition are key elements in the early diagnosis and treatment of sarcopenia. Nutrition-related biomarkers (such as vitamin D, albumin, creatinine, and IGF-1) can be used to assess a patient's nutritional status as well as muscle mass, strength, and function to prevent and treat sarcopenia. However, additional studies are needed to further investigate other candidate biomarkers for sarcopenia.

Applications to Prognosis, Other Diseases, and Conditions

In this chapter, nutritional biomarkers, to prevent and diagnose sarcopenia, have been reviewed. Malnutrition is frequently observed and leads to sarcopenia in addition to other predisposing factors. Furthermore, sarcopenia has become more complicated involving multiple pathogenesis mechanisms, including endocrine dysfunction, growth factors, neuromuscular junction, muscle protein changes, and inflammatory conditions (Curcio et al. 2016). Due to the multifactorial genesis of sarcopenia, it is crucial to emphasize the importance of identifying different biomarkers. The combined use of biomarkers provides greater diagnostic accuracy than individual biomarkers and can be used effectively for the early prognosis of sarcopenia (Kwak et al. 2018).

Nutrition-related biomarkers (such as vitamin D, albumin, creatinine, and IGF-1) can be used to assess a patient's nutritional status as well as muscle mass, strength, and function to prevent or treat the development of sarcopenia. Vitamin D is strongly associated with muscle strength and physical performance as it affects the number and diameter of type II muscle cells. On the other hand, Vitamin D deficiency is associated with chronic diseases, such as cancer (Kilkkinen et al. 2008) and Parkinson's Disease (PD) (Knekt et al. 2010). Vitamin D status acts as a useful biomarker for predicting total mortality, hip fractures, early death, and the development of sarcopenia (Caristia et al. 2019).

Key Facts of Sarcopenia Diagnosis Based on European Working Group of Sarcopenia in Older People (2019)

- Low muscle strength is the main predictor of probable sarcopenia.
- Sarcopenia diagnosis is confirmed by low muscle quantity or quality.
- If low muscle mass, low muscle quantity or quality, and poor physical performance coexist, sarcopenia is severe.
- DXA and BIA methods are recommended for the measurement of muscle quantity in clinical practice.
- Sarcopenia presented for less than 6 months is considered an "acute condition." Sarcopenia presented for $\geq$6 months is considered a "chronic condition."
- Creatinine excretion rate is a promising surrogate measure for assessing whole-body muscle mass.

Mini Dictionary of Terms

- **Malnutrition** refers to the deficiency or imbalance in the intake of energy and/or nutrients by an individual. "Malnutrition" includes stunting, wasting, underweight, and nutrient deficiencies or insufficiencies. On the other hand, malnutrition, also, refers to an excess in the intake of energy and/or nutrients by an individual.
- **Muscle quality** is the microscopic and macroscopic changes in muscle structure and composition, as well as in muscle function provided per unit.
- **Skeletal muscles** (or voluntary muscles) allow humans to move and perform daily activities. Skeletal muscles are connected to the bones by tendons and produce all the movements of the body parts. The muscle cells of skeletal muscles are known as muscle fibers. Skeletal muscle fibers are broadly classified into "slow-contracting" (type I) and "fast-contracting" (type II).
- **Type I fibers** are slow-twitch muscle fibers, and they are smaller than the type II fibers. They are particularly important for prolonged submaximal (aerobic) exercise activities, and they are also more resistant to fatigue. Type I fibers produce less force, but they are able to maintain longer-term contractions, a key for stabilization.
- **Type II fibers** are characterized by high power, high strength, and high-speed production. They are an important factor in strength activities, but they tire more quickly than type I fibers. They are further subdivided into type IIB and type IIA. Type IIB fibers produce the greatest strength but are highly dependent on anaerobic metabolism. Type IIA fibers are capable of using both aerobic and anaerobic energy systems. These fibers have a higher oxidative capacity and fatigue more slowly than type IIB fibers.
- **Visceral proteins** are mainly synthesized in the liver and are fast turnover proteins. Low serum levels indicate protein energy malnutrition (PEM), inflammatory state, and reduced synthetic capacity of the liver.

Summary Points

- The prevalence of sarcopenia varies according to a) the characteristics (race, ethnic differences in body composition) and the living situation of the study population (hospitalized, community-dwelling, and nursing home residents), b) the diagnostic criteria, and c) the methods used to estimate muscle mass, strength, and physical performance. For example, the prevalence of sarcopenia was 51% and 31% men and women, respectively, in nursing homes, while between hospitalized individuals it was 23% for men and 24% for women.
- Among the risk factors of sarcopenia are third age, low level of physical activity, malnutrition, body mass index (BMI), and several comorbidities, such as cardiovascular diseases, diabetes, respiratory diseases, human immunodeficiency virus (HIV), and cancer.
- Malnutrition is very important in the development of sarcopenia. As a result, a plethora of biomarkers are suggested to assist in the diagnosis of sarcopenia, such as vitamin D, leptin, creatinine, and IGF-1.
- A variety of biomarkers, such as inflammatory, neuromuscular, and hormonal biomarkers and biomarkers related to protein metabolism, have been investigated for their role in the early diagnosis of sarcopenia.
- Vitamin D status acts as a useful biomarker for predicting total mortality, hip fractures, early death, and the development of sarcopenia.
- Adipose tissue secretes leptin and proinflammatory cytokines, which stimulate muscle catabolism, thus triggering a vicious cycle that leads more rapidly to sarcopenia, further weight gain mainly in the form of fat, and eventually, physical disability.
- Creatinine is a reliable biomarker for muscle mass because of its easy accessibility and cost-effectiveness.
- Low concentration of IGF-1 is associated with skeletal muscle mass loss, which probably plays a crucial role in the development of sarcopenia.

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Bone Density Measurements and Biomarkers in Nutrition: DXA (Dual X-ray Absorptiometry), Osteopenia, and Osteoporosis

51

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Abstract

The continuous interest of researchers for osteopenia, as well as osteoporosis, has resulted in an enrichment of existing knowledge with new scientific findings. The purpose of this chapter is to provide a concise and critical summary of published research literature concerning the epidemiology, etiology, bone physiology and pathophysiology, clinical manifestations and diagnosis, and prevention and management of these "insidious" diseases. While osteopenia is a clinical condition where bone mineral density is below normal, osteoporosis is a severe systemic

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skeletal disorder characterized by decreased bone mass and disruption of bone microarchitecture resulting in reduced endurance, fragility, and propensity to fracture. Osteopenia and osteoporosis are age related and manifest more often after menopause in women and later in men. Osteoporosis can be compared to other serious health problems, because bone fractures are associated with a high degree of morbidity, mortality, and disability. Fractures have usually impairment in activities of daily living and, not uncommonly, are the beginning of an institutionalized life. Clinically, the symptoms are bone deformation and diffused pain, especially in the spine, but osteoporosis and osteopenia could be asymptomatic. In the last 15 years, osteoporosis has become a major focus. The first step for an efficacious cure is the early diagnosis through measurement of bone density with DXA, before fracture risk becomes too high. Accordingly, prevention of bone fractures is of utmost importance at advanced age and is directly related to bone health. Optimization of bone condition must be the main concern throughout life for both men and women. Stepping-stone for this is the regulation of all the parameters that affect vitally the quality of life, mostly nutrition and exercise.

Keywords

Osteoporosis · Osteopenia · Bone mineral density · Bone mass · Nutrition · Biomarkers · DXA

Abbreviations

BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
DXA	Dual-energy X-ray absorptiometry
NOF	National Osteoporosis Foundation
QCT	Quantitative computed tomography
WHO	World Health Organization

Introduction

Osteopenia and osteoporosis, both defined as states of abnormally low bone mineral density (BMD), are common musculoskeletal disorders, especially in the elderly. In particular, osteopenia is a clinical term that describes a decrease in bone mineral density below normal reference values (but not low enough to meet the diagnostic criteria for osteoporosis) (Table 1). As defined by the World Health Organization (2007), a T-score between −1 and −2.5 defines osteopenia (while values less than −2.5 are diagnostic for osteoporosis) (Table 2). Decreasing BMD values are indicative of an underlying disruption in the microarchitecture of bone and quantitative disorder of bone mineralization (Porter and Varacallo 2021). A combination of physical, metabolic, and/or endocrine factors is considered to be the cause for osteopenia (Raisz 2005). Generally, decreased daily living activities reduce the

Table 1 Differences between osteopenia and osteoporosis

Osteopenia	Osteoporosis
Low bone mass below normal	Too low bone mass
$-1 > T > -2.5$	$T \leq -2.5$
Higher prevalence than osteoporosis	Lower prevalence than osteopenia
Low fracture risk	High fracture risk
May progress to osteoporosis	Osteoporosis is established
No fragility fracture	Presence of fragility fracture in severe cases
Medication is necessary only in the presence of risk factors	Medication is always necessary

Table 2 Diagnostic criteria for osteoporosis

Classification	*T-score*
Normal	$T \geq -1$ (SD*)
Osteopenia	$-1 > T > -2.5$
Osteoporosis	$T \leq -2.5$
Severe (or established) osteoporosis	≤ -2.5 and osteoporotic fragility fracture

[a]SD = standard deviation below the young adult female reference mean

mechanical loading to both bone and skeletal muscle and may result in a decrease of BMD as osteopenia (Toshima et al. 2018).

Conversely, osteoporosis is a severe systemic skeletal disorder characterized by reduced bone mass, microarchitectural deterioration of bone tissue, and a propensity to fracture (National Osteoporosis Foundation [NOF] 2013). Osteoporosis is one of the major and growing healthcare problems around the world, largely related to the general aging of societies (World Health Organization [WHO] 2007). In the last 15 years, age-related osteoporosis has become a major focus, and significant progress has been made both in defining this disorder and in understanding its complex pathogenesis. Most importantly, a consensus has emerged concerning the strength of the association between low bone mineral density and fracture risk (Kanis, 1994, 2002; NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001).

Osteoporotic bone fractures are associated with a severe degree of disability and a high direct cost for rehabilitation and have an adverse impact on mortality (Cole et al. 2008a; WHO 2007). Thus, prevention of osteopenia and osteoporosis is of utmost importance, especially for the elderly.

The purpose of this chapter is to provide a concise and critical summary of published research literature concerning the epidemiology, etiology, bone physiology and pathophysiology, clinical manifestations and diagnosis, and prevention and management of osteopenia and osteoporosis. This will help researchers, physicians, and other health professionals to keep current on the evidenced-based research about theses "insidious" diseases.

Epidemiology

Osteoporosis affects more than 75 million people in the USA, Europe, and Japan, while worldwide it is involved in approximately 8 to 9 million fractures annually, of which 4,5 million were in the USA and Europe (WHO 2007). Osteoporotic fractures account for 0.83% of the global burden of non-communicable disease, and the 1.75% of the global burden is in Europe (Johnell and Kanis 2006). In 2000, the total disability-adjusted life years (DALYs) lost were 5.8 million, of which 51% were due to osteoporotic fractures that occurred in Europe and the Americas. The annual financial cost in Europe (approximately 48 billion dollars) is higher than that of cancer or cardiovascular diseases (Kanis and Johnell 2005).

Epidemiologic studies have subsequently provided insight into the prevalence of osteoporosis in the elderly population (Cole et al. 2008a; Umland 2008). Although fractures of the hip, wrist, and spine are often focused upon almost any bone can fracture, virtually, all fractures in the elderly can be attributed to osteoporosis, whether primary or secondary, and evidently, the occurrence of these fractures increases exponentially with age (Gooren 2007; WHO 2007).

Osteoporosis affects both women and men and has an impact comparable to, if not greater than, the major health problems, such as cardiovascular disease and malignancy. The female-to-male ratio of hip fractures is approximately 2:1 (DeLaet and Pols 2000; NOF 2013). Men, probably due to their larger accrual of bone mass in puberty, suffer bone fractures approximately 10 years later in life than their female counterparts, and using a delay of approximately 10 years for comparison with women, incidence and prevalence rates in men are not very different from the rates in women. But, men's clinical condition at that age has usually also deteriorated, and it is not surprising that morbidity associated with fractures and their (surgical) treatment is considerably greater than in women.

Morbidity and mortality following bone fracture have been the focus of many research efforts carried out over the last few decades (Tosteson et al. 2007). These endeavors concern mainly the hip fractures, which have a detrimental effect on quality of life, after only 30% of the elderly retrieves the level of functionality that was before fracture. Almost 1/3 of patients with hip osteoporotic fracture are hospitalized even for 1 year, and it is remarkable that one of five patients departs within the first year after the fracture. Fractures in hips and the vertebrae occur mainly in women aged over 70 or 80 years, and fractures in the wrist occur mostly in women between 50 and 70 years, while all other fractures, such as in the pelvis, can occur throughout life after menopause (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001).

Osteoporosis has become a major problem even in developing countries. The prevalence of osteoporosis and incidence of fracture vary by gender and race/ethnicity. The incidence of hip fractures in relation with age is higher in Caucasians than in Asians, but there is a lot of variation even between the different communities of the same race (Eisman 1999; NOF 2013). The probability for a 50-year-old white woman or man to sustain hip fracture, during lifetime, is 14% and 5–6%, respectively. Over 50% of postmenopausal white women will have an osteoporotic-related fracture (Porter and Varacallo 2021). The risk for African Americans is very less, 6%

and 3% for a 50-year-old woman or man, respectively (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001). The difference in frequency between ethnic and racial groups may be related to environmental factors but can also reflect heritable differences in vulnerability.

Etiology

From birth to adulthood, there is a bone mineral acquisition that follows a predictable trend specific to an individual's age and sex. During puberty, bone mineral gain increases to its maximum level, and this bone mineral accretion rate remains the greatest for males and females for about 4 years after the peak gain rate is achieved. The 95% of adult bone mass is achieved by age 17 for females and 21 for males; thus, peak bone mass is normally achieved by the third decade of life. After the age of 30, there is a gradual and natural bone mass reduction that continues for the later life (Weaver et al. 2016). Failure to reach peak bone mass in adolescence and adulthood results in early-onset conditions of decreased bone mass (or osteopenia) and increased risk of osteoporosis and fragility fractures in later life.

Heritable factors contribute up to 80% to the ability to attain and maintain optimal bone mineral density level (Varacallo et al. 2021). Natural bone mass reduction in adulthood occurs gradually due to modifiable factors which include body mass, nutrition status (adequate calcium and vitamin D daily intake), weight-bearing exercises, and hormonal status. This natural bone loss is considered to be the cause of primary forms of osteopenia and osteoporosis, while secondary causes accelerate this process (including lifestyle factors like smoking, abuse of alcohol, and sedentary lifestyle).

Primary osteoporosis is related to the aging process in conjunction with decreasing sex hormones. There is deterioration in bone microarchitecture, leading to loss of bone mineral density. Secondary osteoporosis and low bone mass state (osteopenia) are caused by other diseases or their treatments (e.g., hyperthyroidism, hyperparathyroidism, anorexia, malabsorption syndromes, hypogonadism, chronic renal failure, Cushing syndrome, non-estrogen hormonal therapy, secondary amenorrhea/oligomenorrhea). White or Asian race, low body weight (BMI under 18.5 kg/m^2), smoking, family history, early menopause, calcium and/or vitamin D deficiency, low levels of physical activity, and any disease or condition that can affect overall mobility level with long-term immobilization (such as spinal cord injuries) can also lead to rapid loss of bone mass and are risk factors for osteoporosis (Varacallo and Fox 2014) (Table 3). Also, medications like glucocorticoids, long-term steroid use, anti-epileptics, and chemotherapy agents can lead to secondary osteoporosis or are suspected to contribute to osteopenia and osteoporosis (Varacallo et al. 2021).

Bone Physiology and Pathophysiology

Bone is a complex organ. It contains an organic matrix that serves as frame and calcium as mineral distributed in a pattern, which provides structure and serves as an ion stock for the body. Within this complex tissue reside specialized bone cells,

Table 3 Risk factors for osteoporosis and fragility fracture

Gender (female > male)
Asian or Caucasian race
Aging
Low peak bone mass
Low BMI
Thyroid diseases
Vitamin D deficiency
Low calcium intake
Early menopause
Primary or secondary amenorrhea/oligomenorrhea
Hypogonadism
Neuromuscular disorders
Abuse of alcohol
Smoking
Medications (e.g., glucocorticoids)
Low levels of physical activity or immobilization

including osteoblasts, osteocytes, and osteoclasts (Seeman and Delmas 2006). Bone is also a dynamic organ and continually models-remodels itself throughout life ("bone modeling-remodeling"). This process involves removal or resorption of bone from one surface of bone and the subsequent deposition of new bone on another nearby surface. These two specific actions, bone resorption and formation, are performed by the specialized bone cells, and these events are tightly coupled in time and space (Becker 2006; Umland 2008).

In osteoporosis, there is a decrease in unit volume in both the organic part of bone and calcium, without changing the ratio of one to another, unlike in osteomalacia. Hence, the composition and volume of bone remain normal, but the bone mass in proportion to volume, the thickness of cortical bone, and the number and size of trabeculae in cancellous bone decrease (bone trabeculae with normal composition but sparser), as well as the connections among them, thereby impairing the bone microarchitecture (Simon, 2005). Eventually, osteoporosis leads to bone with diminished tensile strength and significantly more susceptibility to fracture with less force. At some point, the amount of bone available for mechanical support falls below a certain threshold, and the patient may sustain a fracture.

Many factors, more or less interdependent on each other, participate in the setting of the above procedures and influencing the accumulation of bone mass during growth (Raisz 2005). These factors may be genetic, which quantitatively seems to be the most important, dietary (calcium, phosphorus, proteins), hormonal (sex steroids, calcitonin, factor IGF-1), mechanical (exercise, body weight), and also the gender, race, and exposure to risk factors (Frost 1997; Becker 2006; Rizzoli et al. 2001). Most of these factors are involved in both the maintenance of bone mass in adulthood and loss of bone later, although in different proportions compared to their role in the acquisition of peak bone mass.

Estrogens, the female sex hormones, appear to be necessary not only for both men and women to reach the peak bone mass during puberty but also for its preservation in adulthood. These hormones control bone remodeling during reproductive life in females and later on in elderly men (Weitzmann and Pacific 2006). Pathologic conditions associated with premature estrogen deficiency (e.g., anorexia nervosa, secondary amenorrhea due to arduous exercise) further support the concept of a causal link between estrogen inadequacy and increased bone loss (Rizzoli et al. 2001). Estrogen deficiency is the main cause of postmenopausal osteoporosis and probably plays an important role in male osteoporosis. Consequently, estrogen deficiency is directly implicated in the age-related increase in the incidence of fractures (Riggs et al. 2002).

Vitamin D is important in the maintenance of skeleton integrity in adults. Elderly people tend to have poor dairy calcium and vitamin D intakes and decreased sunlight exposure and dermal production of vitamin D. Also, vitamin D and calcium supplementation has been demonstrated to significantly increase BMD and decrease the incidence of osteoporotic fractures in the elderly (Bischoff-Ferrari et al. 2006; Jackson et al. 2006).

In the matter of bone mass, there is no consistent difference between genders before puberty, at any skeletal site (Rizzoli et al. 2010). On the contrary, there is no evidence for a gender-related difference in bone mineral density at birth, and this sameness in bone mass between males and females is maintained until the onset of pubertal maturation. In adolescence, bone mineral mass of various skeletal sites, such as the lumbar spine, doubles, and this increase occurs approximately 2 years earlier in females than in males. Meantime, a gender-related difference in peak bone mass becomes detectable and appears to result essentially from a longer period of bone mass gain in males than in females, resulting in a larger increase in bone size and cortical thickness in the former (Seeman 1997). Thus, at the end of puberty, the peak bone mineral content at the lumbar spine and the proximal femur is higher in males than in females, while bone mineral density does not differ significantly (Gilsanz et al. 1988, 1997).

The gain in length and the gain in bone mass do not occur simultaneously. The peak of growth in stature precedes the peak of maximal bone mass gain. In males, the greatest difference occurs in the age of 13–14 years and is more pronounced for the lumbar spine and femoral neck than for the mid-femoral shaft, while in females, it occurs in the age of 11–12 years (Theintz et al. 1992; Fournier et al. 1997). Peak bone mass is reached by men and women about in the middle of the third decade of life, is probably genetically predetermined, and remains constant in the subsequent plateau period of bone turnover, with equal rates of bone formation and resorption (Riggs et al. 2002; Mora and Gilsanz 2003). This lifelong and dynamic remodeling process in the adult skeleton occurs on all bone surfaces, including the periosteal, trabecular, cortical, and endosteal surfaces, in order to maintain the strength of bone (Bonjour et al. 2007).

Following the aforementioned plateau phase, a period of net bone loss equivalent to about 0.3% to 0.5% per year begins for both genders (Simon 2005; Becker 2006). Beginning with the decrease in estrogen in association with the menopause, women

accelerate this net bone loss about tenfold for approximately 5–7 years. The steady loss of bone affects equally men and women after the age of 70 years, while dominant problem remains the reduced production of new bone (The North American Menopausal Society [NAMS] 2010).

Bone size varies little throughout life, except a slight expansion of bone cortex, mainly in men. This periosteal expansion is less than the increase in bone marrow space due to endosteal resorption, which increases with age in both genders, resulting in a thinner bone cortex. These conditions, combined with an increment in cortical bone and a destruction of trabecular bone, account for the age-dependent bone loss. It is well documented that bone loss does not drop with age, but continues throughout life, at least in peripheral skeletal sites (Ensrud et al. 1995; Seeman 1997; Rizzoli et al. 2001).

The strength of bone depends on the total size, the volume and density, as well as its structural characteristics. The total bone mass of each person in each phase of his life depends on the amount of bone formed during adolescence or even in the third decade and by the subsequent loss due to aging and menopause (Riggs et al. 2002; Mora and Gilsanz 2003). Usually, there is a misperception that osteoporosis is always the result of bone loss that commonly occurs in men and women because of aging. However, someone who does not reach its optimal, peak bone mass during childhood and adolescence may develop osteoporosis without the occurrence of increased bone loss. Therefore, sub-optimal bone growth before adulthood is as important as bone loss to the development of osteoporosis (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001). The factors that determine the peak bone mass, bone turnover, and loss of bone are the main objectives wherein the research of osteoporosis focuses.

Clinical Manifestations and Diagnosis

Clinically, the symptoms of osteoporosis manifest mostly in women after menopause with main characteristic the deformities, loss of height, and widespread pain in bones but could be also asymptomatic (Becker 2006). Sometimes, pain is more acute, and movements in the vertebral column are limited and painful, a condition that reveals compressive fractures of the vertebrae, largely in low thoracic and lumbar spine. The typical osteoporotic fractures occur suddenly and sometimes after a fall, sudden movement, weight-lifting, jump, or even cough. The pain is chronic and may be severe and is typically located mainly at the region of fracture but may radiate to the abdomen or flanks. Elderly over 65 years of age suffer mainly from fractures of the femoral neck, and incidence increases as people age. Most patients with osteoporotic fracture sustain other fractures within the next few years (Simon 2005; NAMS 2010).

Osteoporosis can occur as a primary disorder or as a disorder associated with various diseases (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001). Primary osteoporosis is a disease of the elderly, particularly among older women, with most cases occurring in the sixth and later decades of life. This form of osteoporosis is sometimes referred to as “involutional”

osteoporosis. Secondary osteoporosis is a series of abnormalities and diseases that may manifest with effects in bone. Those disease states associated with osteoporosis include endocrine disorders, systemic inflammatory diseases (like rheumatoid arthritis), bone mineral and metabolic defects, and other chronic illnesses.

The aims of screening the individuals who are at risk for osteoporosis is to put the diagnosis on the basis of the assessment of bone mass, to determine fracture risk, and to take decisions regarding the appropriate treatment. Typical radiological examinations permit identification of architecture of bone. In reality, however, the X-rays, although inexpensive and simple, allow only partial quantitative evaluation of bone mass and provide little information for the cancellous bone, which is the most active metabolically and is involved in postmenopausal osteoporosis. For that reason, more appropriate methods have been developed for quantifying both the cortical and trabecular bone mass and assessing fracture risk (Simon 2005).

Dual-energy X-ray absorptiometry, abbreviated as "DXA" (although usually abbreviated in older literature as "DEXA"), was first introduced in 1987 (Hologic QDR-1000 system, Hologic, Inc) and immediately made all previous forms of radiation-based BMD measurement systems obsolete (Miller 2017). Since then, there have been many generations of the technology, with the main US manufacturers in 2017 being Hologic, Inc. and GE Lunar.

Nowadays, DXA is recognized as the reference method ("gold standard") to measure bone mineral density with acceptable accuracy errors, good precision, and reproducibility. The examination with DXA has become the best choice for measurement and accurate diagnosis of osteoporosis as this technique is used for measuring bone mass and density and identifies individuals whose osteoporosis is so severe as to be qualified at potential fracture risk (NOF 2013) (Table 4). It is also used to monitor patients undergoing treatment by performing serial assessments. Areal BMD is expressed in absolute terms of grams of mineral per square centimeter scanned (g/cm^2). The measurement of bone mass is also provided by DXA technique through the expression of bone mineral content (BMC) in terms of grams (g).

The diagnosis of normal, low bone mass (or osteopenia), osteoporosis, and severe or established osteoporosis is based on the WHO's diagnostic classification through BMD (WHO 2007). By using population standards, osteoporosis is defined by BMD at the hip or spine that is less than or equal to 2.5 standard deviations (*SD*) below the mean of a reference population of young, normal, white women ("*T*-score"). In the same manner, osteopenia is defined as a bone density between 1.0 and 2.5 *SD*s below

Table 4 Why BMD measurement with DXA ("gold standard") for diagnosis of osteoporosis?

Osteoporosis is a severe systemic disorder
Osteoporosis causes fragility fractures
Osteoporosis is associated with increased morbidity, hospitalization, and mortality
Easy to put early the diagnosis of osteoporosis
Prevention and reduction in fracture risk with early management and treatment
Low cost
Low level of radiation

Table 5 Indications for BMD measurement

Women ≥65 yrs of age
Men ≥70 yrs of age
Postmenopausal women <65 yrs of age with risk factors
Adults with osteoporotic fragility fracture
Adults with a disease or condition for secondary osteoporosis
Adults under treatment that causes bone loss (e.g., glucocorticoid therapy)
Individual under treatment for low bone mass or bone loss ("follow-up")
Individual not receiving therapy in whom evidence of bone loss would lead to treatment

the bone density of the same reference population. Thus, patients do not have to sustain a fracture to be diagnosed with this insidious problem. Additionally, another norm, the "*Z*-score," provides similar information regarding a patient's BMD in relation to age-matched controls. Thence, with this calculation, it is possible to screen for prominent causes of accelerated bone loss. WHO has established DXA as the best densitometric technique for assessing BMD in postmenopausal women and has based the definitions of osteopenia and osteoporosis on its results (Table 5).

However, currently, there is no accurate measure of overall bone strength. The BMD is frequently used as a proxy measure and accounts for approximately 70% of bone strength; therefore, it might be an excellent predictor of future fracture risk. There is an exponential correlation between the decrease in BMD and increase in fracture risk. Usually, 1 *SD* equals 10–15% of the BMD value in g/cm^2 (NOF 2013). Almost all population studies have now confirmed that for a single *SD* below young normal mean BMD (at virtually any skeletal site), there is a nearly twofold greater risk of an eventual hip fracture (Kanis 1994, 2002; WHO 2007; Kanis et al. 2008).

According to the point of view of the "Utah Paradigm," regarding updated bone physiology, "whole-bone" strength ranks above bone "mass" in physiologic importance and depends strongly on the amount and kind of bone tissue in a bone, the distribution of bone tissue, and the bone's longitudinal and cross-sectional size and shape (Frost 2003b). The measurement with DXA can evaluate the bone "mass" factor in terms of BMC and BMD. However, bone "mass" alone cannot reliably evaluate "whole-bone" strength; hence, currently popular BMD values provide very unreliable indicators of whole-bone strength and a poor evaluation of it, weakening many arguments that depend on such BMD data. Conversely, another method, the quantitative computed tomography (QCT), can evaluate both the "mass" and "architectural" factors in whole-bone strength.

Although available technologies (e.g., peripheral DXA (pDXA), QCT, peripheral QCT (pQCT), quantitative ultrasound (QUS)) measuring central (spine and hip) and peripheral (forearm, heel, fingers) skeletal sites provide site-specific and global (overall risk at any skeletal site) assessment of future fracture risk, DXA measurement at the hip is the best predictor of future hip fracture risk (Singer 2006). However, it is not clear how to apply the diagnostic criterion of *T-score* to men, to children, and across ethnic groups. Because of the difficulty in accurate measurement and standardization between instruments and sites, controversy exists among

experts regarding the continued use of this diagnostic criterion (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy 2001). In accordance with the guidance of the National Osteoporosis Foundation (NOF 2013), the WHO bone mineral density diagnostic classification should not be applied in premenopausal women, men less than 50 years of age, and children (NAMS 2010). In these groups, the diagnosis of osteoporosis should not be made based on densitometric criteria alone (see also the recommendations of the International Society for Clinical Densitometry [ISCD] in ISCD 2007).

Prevention and Management

Osteoporosis and osteoporotic bone fractures are associated with a severe degree of disability, and also their impact on mortality cannot be ignored (Tosteson et al. 2007; Cole et al. 2008a). Furthermore, fractures are usually a forewarning of a handicapped life with impairment in activities of daily living and, sometimes, the beginning of an institutionalized life (Gehlbach et al. 2007; Umland 2008).

Therefore, prevention of osteopenia and osteoporosis and bone fractures is of utmost importance, mostly at advanced age. Patient awareness of the potential risks associated with falling down is also important. Many elderly people are at risk for falling as a result of poor coordination, poor vision, muscle weakness, confusion, and the use of hypnotics or other medications (Cole et al. 2008b). Because they are at a higher risk of falling, they are also at an increased risk of sustaining a fracture.

Osteopenia and osteoporosis are to a great extent preventable and treatable, because of significant scientific progress in the field of diagnosis and treatment and in understanding of their pathogenesis. It must be kept in mind that in the case of osteoporosis, there is no complete cure in the meaning of bringing back the bone mass in normal levels. The first step for an effective treatment is an appropriate diagnosis. Because there are no warning signs prior to a fracture, many people are not being diagnosed in time to receive effective therapy during the early phase of the disease. The goal is to diagnose the patients with osteopenia and osteoporosis before they have sustained enough bone loss to be at risk for a fracture (Cole et al. 2008b). Therefore, people who are at risk for future fracture should be identified early, based on their family history and other known risk factors. Accordingly, they should undergo a bone densitometry examination to determine their bone mass (WHO 2007).

Many factors have been associated with an increased risk of osteoporosis-related fracture. Since the majority of osteoporosis-related fractures result from falls, it is also important to evaluate risk factors for falling (such as a personal history of falling, along with muscle weakness and gait, balance, and visual deficits). Consequently, strategies to reduce falls are essential and should include, but are not limited to, checking and correcting vision and hearing, evaluating any neurological problems, reviewing prescription medications for side effects that may affect balance, and providing a checklist for improving safety at home (NOF 2013).

The main therapeutic goal is the retardation of the disease's progress by decreasing bone resorption and increasing the bone mass. The treatment strategies include

Table 6 Nutritional factors in osteoporosis

Calcium
Vitamin D
Phosphorus
Vitamins K, C, B12, and B6 and folic acid
Proteins
Magnesium
Sodium chloride
Caffeine
Fluoride
Boron
Minerals (zinc, copper, potassium)

medications, such as calcium and vitamin D supplementation, hormone replacement therapy (e.g., estrogens), calcitonin, and bisphosphonates (Simon 2005; Gooren 2007). A good and adequate nutrition is essential for normal growth and also important for all individuals with osteopenia and osteoporosis (Table 6). A balanced diet with the adequate calories and appropriate nutrients is the foundation for the development of all tissues, including bone. Controlled clinical trials have demonstrated that an optimal diet for bone health (for patients older than age 50) must include a sufficient intake of supplemental calcium (at least 1200 mg per day) and vitamin D (800 to 1000 IU per day) and this combination can reduce the risk of fracture (NOF 2013). Additionally, it is recommended to abstain from smoking and the abuse of alcohol and caffeine.

Another therapeutic goal is also the treatment of pain; so, medications for this purpose are useful (e.g., analgesics, anti-inflammatory, and muscle relaxants). The role of physical therapy is also important in pain management, improving muscle function, and reducing the risk of falls by the use of electrotherapy, hydrotherapy, and mild heat and with appropriately adapted exercises of regular weight-bearing and muscle-strengthening (Lange and Uhlemann 2008; Preisinger 2009; Bautmans et al. 2010; Bennell et al. 2010; Dusdal et al. 2011; Lange et al. 2012). For example, special therapeutic exercise programs for dorsal muscle-strengthening in women with kyphosis and lumbar spine vertebral fractures had positive effects in muscle force, mobility, reducing pain, better sleep, and quality of life (Hongo et al. 2007; Qvist et al. 2011).

Physical activity, in general, can play an important role in the prevention and treatment of osteopenia and osteoporosis. A key factor for the bone mass and density is the mechanical loading applied to the bone, which is achieved by special physical activity that involves exercises of weight transfer (Bailey and Brooke-Wavell 2008). In fact, active people with intense daily activity have greater bone density than non-active persons, while the lack of mechanical loading and the long lying-in bed have devastating effects on bones (Zhang et al. 2008; Humphries et al. 2000; Rizzoli et al. 2010). The specific benefits of lifelong physical activity on bone health have been investigated in numerous randomized clinical trials and observational studies (Kohrt et al. 2004; Vicente-Rodriguez 2006; Marcu et al. 2010).

Some evidence indicates that resistance and high-impact exercise are likely the most beneficial (Martyn-St James and Carroll 2009; Vicente-Rodriguez et al. 2007). Moreover, there is convincing evidence that exercise in elderly people also improves function and delays loss of independence by increasing muscle mass and strength, agility, body posture, and balance and thus contributes to quality of life by reducing the risk of falls (approximately 25%) and possibly fracture risk (Cole et al. 2008b; de Kam et al. 2009; Guadalupe-Grau et al. 2009).

However, the most important parameter in the treatment of osteoporosis is prevention, which is directly related to bone health. Optimization of bone health is a process that must occur during lifetime of both men and women. Factors that affect the good condition of the bones at all ages are essential for the prevention of osteoporosis and its consequences. Therefore, prevention strategies should be based on the formation of these determinants, especially those involved in bone mineral density and its changes with increasing age (Vicente-Rodriguez et al. 2008). Consequently, the most important determinant of bone health is the peak bone mass that is achieved until the third decade of life. Of course, genetic factors play a strong and dominant role on peak bone mass, but the modifiable factors that are related to quality of life, such as nutrition, diet, and physical activity, are also crucial. Moreover, childhood is a critical period for adopting habits and lifestyles which promote the preservation of the good condition of the bones. For example, smoking that usually starts in adolescence can have a deleterious effect on the acquisition of peak bone mass. Proper and balanced diet with enough calories and nutrients, with the calcium being in predominant position, is also essential for normal development.

Overall, the implementation of specific exercise programs in critical younger ages helps the achievement of the ideal peak bone mass (Wang et al. 2007; Pate et al. 2010; Gracia-Marco et al. 2011a, 2011b). This enables a substantial advantage to individuals who are at risk of osteopenia, since the bone loss, though it will increase due to the disorder, will start by higher prices for bone density having as a result the reduced negative consequences of osteoporosis (Rizzoli et al. 2010).

Finally, according to the still-evolving skeletal-biologic Utah Paradigm, there are some different aspects regarding osteoporosis, namely, its pathophysiology and biomechanical pathogenesis, classification, diagnosis, prevention, management, and treatment. For the paradigm's specific suggestions and further definitions in reference with osteopenia and osteoporosis, there is a specialized bibliography by Harold M. Frost (1985, 1987, 1991, 1992, 1994, 1997, 1998, 1999, 2001a, 2001b, 2003a, 2003c), José Luis Ferretti, and Webster S. S. Jee (Ferretti et al. 1995, 2003; Frost et al. 1998; Jee 2000, 2006).

Mini-Dictionary of Terms

- Biomarker (A portmanteau of "biological marker") = A broad subcategory of medical signs – that is, objective indications of biological state observed from outside the body – which can be measured accurately and reproducibly.

- Body Mass Index = A person's weight in kilograms divided by the square of height in meters.
- Bone Mass = A function of bone size and volumetric bone mineral density.
- Bone Mineral Content (BMC) = A measurement of the amount of minerals (mostly calcium and phosphorus) contained in a specific area of bone.
- Bone Mineral Density (BMD) = A measure of the amount of minerals (mostly calcium and phosphorus) contained in a certain volume of bone.
- Dual-Energy X-ray Absorptiometry (DXA or DEXA) = An imaging technique that measures bone mineral density by passing X-rays with two different energy levels through the bone.
- Quantitative Computed Tomography (QCT) = An imaging technique that measures bone mineral density using a standard X-ray computed tomography (CT) scanner with a calibration standard to convert Hounsfield units of the CT image to bone mineral density values.
- Quantitative Ultrasound (QUS) = An imaging technique that measures bone mineral density measuring the velocity and attenuation of ultrasonic sound waves as they pass through bone tissue.
- Osteopenia = A condition where bone mineral density is lower than normal; however, not yet as low as osteoporosis and falls in the "T"-score range of −1.1 to −2.5.
- Osteoporosis = Osteoporosis is a systemic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissues.

Summary Points

- Osteoporotic bone fractures are associated with a severe degree of disability, a high direct cost for rehabilitation, and an adverse impact on mortality.
- Virtually, all fractures in the elderly can be attributed to osteoporosis, whether primary or secondary, and evidently, the occurrence of these fractures increases exponentially with age.
- This natural bone loss is considered to be the cause of primary forms of osteopenia and osteoporosis, while secondary causes accelerate this process (including lifestyle factors like smoking, abuse of alcohol, and sedentary lifestyle).
- The strength of bone depends on the total size, the volume and density, as well as its structural characteristics.
- The typical osteoporotic fractures occur suddenly and sometimes after a fall, sudden movement, weight-lifting, jump, or even cough.
- According to the "Utah Paradigm," "whole-bone" strength ranks above bone "mass" in physiologic importance and depends strongly on the amount and kind of bone tissue in a bone, the distribution of bone tissue, and the bone's longitudinal and cross-sectional size and shape.
- Although available technologies (e.g., peripheral DXA (pDXA), QCT, peripheral QCT (pQCT), quantitative ultrasound (QUS)) measuring central (spine and hip) and peripheral (forearm, heel, fingers) skeletal sites provide site-specific and

global (overall risk at any skeletal site) assessment of future fracture risk, DXA measurement at the hip is the best predictor of future hip fracture risk.

- Osteopenia and osteoporosis are to a great extent preventable and treatable, because of significant scientific progress in the field of diagnosis and treatment and in understanding of their pathogenesis.

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Linking Biomarkers with Causes, Lifestyle Factors, and Management of Sarcopenia

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Abstract

Analysis of a disease or a syndrome, usually, hides metabolic pathways which still remain unexplored. The incidence of sarcopenia makes difficult to find out which specific mechanism provokes this syndrome. Also, the different lifestyle factors and ways in which people live nowadays augment the difficulty to interpretate sarcopenia. Analyzing the lifestyle factors, it was observed that the multiple secretion of biomarkers affects the expression of the syndrome positively or negatively. Lifestyle factors such as physical activity, nutrition, sleep, and abuses can affect the secretion of biomarkers such as tumor necrosis factor, interleukins, and hormones. According to these secretions, sarcopenia patients can be affected positively or negatively. Thus, it is observed an exacerbation or remission on syndrome's symptoms that can affect the quality of life, performance parameters, and muscle mass tissue. Concluding, the management of sarcopenia consists of the contribution of various lifestyle factors. Participating on physical activity programs, learning healthy nutritional habits, and declining the abuses like alcohol cigarettes, etc., patients can effectively deal with this syndrome.

Keywords

Biomarkers · Physical activity · Nutrition · Sleep · Abuses · Macronutrients · Micronutrients · Alcohol · Smoking · Inflammation

Abbreviations

3-MH	3-methylhistidine
4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
Akt	Protein kinase B
BAT	Brown adipose tissue
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CER	Creatinine excretion rate
CO	Carbon monoxide
COHb	Carboxyhemoglobin

COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
DEXA	Dual-energy X-ray absorptiometry
FFM	Fat free mass
FGF 2, 6	Fibroblast growth factor 2, 6
FR	Free radicals
GH	Growth hormone
HPA	Hypothalamic-pituitary-adrenal
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-1RA	Interleukin 1 receptor antagonist
InCHIANTI	Invecchiare in Chianti
KNHANES	Korea National Health and Nutrition Examination Survey
LBM	Lean body mass
MAFbx	Muscle atrophy F-box
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mTOR1	Mammalian target of rapamycin
mTORC1,2	Mammalian target of rapamycin complexes 1,2
MUL1-Akt	Mitochondrial E3 ubiquitin protein ligase 1
MuRF1	Muscle RING-finger protein-1
RBP	Retinol-binding protein
ROS	Radical oxidative stress
S6K	Ribosomal protein S6 kinase
SPPB	Short physical performance battery
TNF-α	Tumor necrosis factor alpha
TUG	Timed up and go
UPS	Ubiquitin–proteasome system
WAT	White adipose tissue
WHO	World Health Organization

Introduction

Muscle Atrophy, Then and Now

Muscle atrophy is one of the most common states of muscle tissue waste. Researches attempt to explain the cause of this phenomenon from the eighteenth century after a sequence of surgeries on animals. In 1921, Lipschütz and Audova (1921), trying to make a step forward about the explanation of muscle atrophy mechanisms, found that muscle tissue atrophy is caused, mainly, due to inactivity. This conclusion came

after the cut of rabbits' sciatic nerve and the Achilles tendon, causing difficulties in their movement because of inactivity.

Nowadays, with the contribution of technology, muscle atrophy and different kind of diseases can be diagnosed via magnetic resonance imaging (MRI) (Weber et al. 2018). Also, studies have been specified and explained this phenomenon through three main categories:

1. Physiologic which is caused with:
   (i) Inactivity
2. Pathologic which is caused with:
   (i) Aging
   (ii) Starvation
   (iii) Diseases
3. Neurogenic which is caused with:
   (i) Injury
   (ii) Disease of a nerve

The common symptom between the pathological conditions of aging, starvation, and diseases is the muscle degradation. Also, each of the three conditions shows many similarities regarding to their symptoms. For example, sarcopenia, which is a syndrome that comes with aging, and cachexia, which is a disease foremost observed in AIDS, cancer, etc., share common characteristics like decreased muscle mass, strength, functionality, and quality of life, which lead to movement slowness (Kim et al. 2021; Shafiee et al. 2017). Thus, in some cases on patients, the scientists and doctors meet difficulties to observe the differences between the pathologic conditions. Hence, the topic of this chapter is to specify the most common knowledge syndrome which is met during elderly and is the "poverty of flesh" or well known as sarcopenia (Irwin et al. 1999).

Symptoms and Mechanism of Sarcopenia

Symptoms

Sarcopenia is a widely *known syndrome which* is met during elderly and described by a progressive loss of skeletal muscle mass and strength (Cruz-Jentoft et al. 2010; Shafiee et al. 2017). Accordingly, through sarcopenia, the muscles degradation causes: instability, weakness, and generally function decline (Cruz-Jentoft et al. 2010). The above symptoms can be predicted through different diagnostic tools like: bioelectrical Impedance analysis (BIA) or dual energy X-ray absorptiometry (DEXA).

BIA and DEXA are some of the most expensive methods (Shafiee et al. 2017). Obliquely and cheaper methods, also can be used, contributing to sarcopenia's valid and reliable identification, are the:

(i) Walking test for 4 m (lower than 0.8 m/s speed)
(ii) Low amount of muscle mass ((% of muscle mass / height)^2)
(iii) Hand-grip strength test
(iv) Chair stand test

For better understanding of the symptoms and the severity of the syndrome, sarcopenia is categorized into: (i) pre-sarcopenia, (ii) sarcopenia, and (iii) severe sarcopenia (Kim et al. 2021) (Table 1). Independently the categorization, sarcopenia is the cause of many other secondary symptoms which mainly affect functionality and individual's quality of life (Table 2).

Table 1 Symptoms of sarcopenia according to categorization

	Categories		
Symptoms	**Pre-sarcopenia**	**Sarcopenia**	**Severe sarcopenia**
Muscle mass	↓	↓	↓
Muscle strength	–	↓	↓
Physical performance	–	–	↓

↓ : Decrement – : Stable

Table 2 Secondary symptoms of sarcopenia

Authors	Type of study	Symptoms
Cannataro et al. (2021)	Review	Loss of skeletal muscle mass and function Disability Metabolic dysfunction Poor quality of life Death
Hu et al. (2021)	Original article	Adverse individual physical and metabolic changes Morbidity Mortality Increased firing rate of motor unit
Kim et al. (2021)	Review	Loss of skeletal muscle mass and function Loss of skeletal muscle strength Physical performance decrement
Shafiee et al. (2017)	Original article	Loss of muscle mass and function Disability Poor quality of life Increased risk of death

Mechanism

Analysis of a disease or a syndrome, usually, shows metabolic pathways which still remain unexplored. Sarcopenia is relatively a new and multifactorial syndrome. The main factors or situations which led to the degradation of muscle tissue are still under research (Kim et al. 2021). Although there is no sufficient explanation, there are many possible proving factors (Kim et al. 2021) such as: satellite cells, inflammation, fibroblast growth factors (FGF), hormonal factors, autophagy, myosteatosis, reactive oxygen species (ROS), p38MAPK, and p16Iu4a.

Each of these factors, autonomy or synergically, affects the muscle mass tissue resulting in muscle loss, decreased function, instability, and generally low quality of life (Kim et al. 2021). The mechanisms behind possible causes of sarcopenia have been analyzed in ▶ Chap. 50, "Candidate Biomarkers for Sarcopenia and Relationship with Nutrition." The aim of this chapter is to try to present the possible connections among mechanisms (Fig. 1).

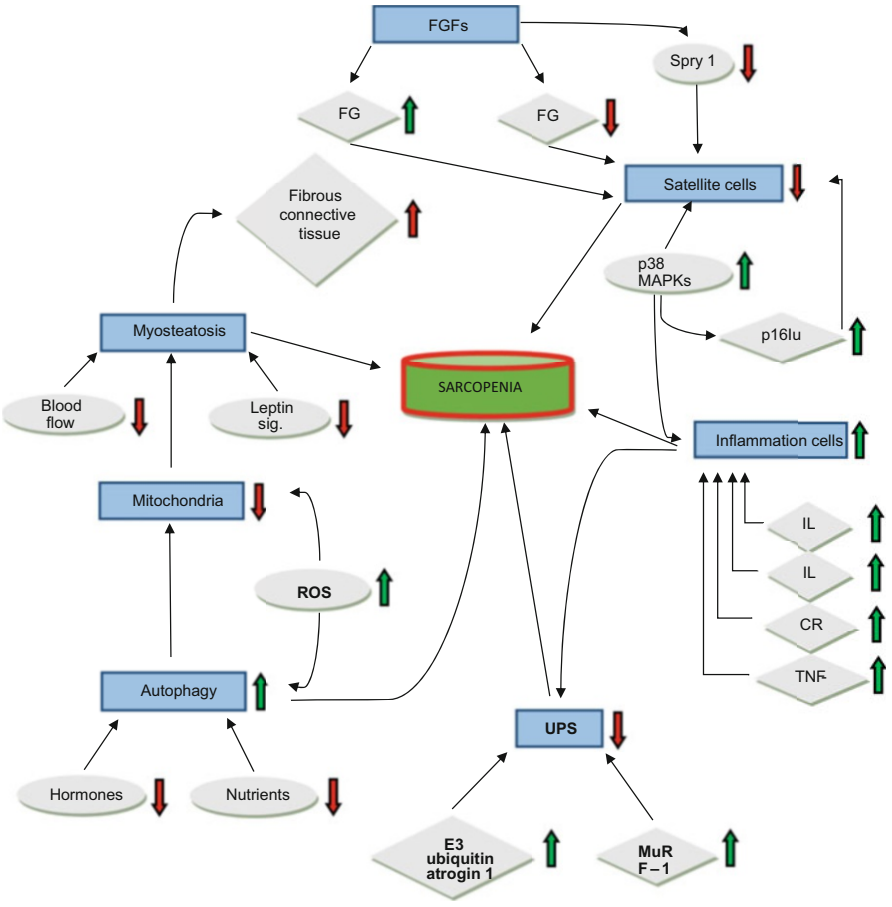

Fig. 1 Pathophysiological pathways of sarcopenia

Inflammatory exacerbation through the increment of interleukins (IL-1, IL-6), C-reactive protein (CRP), and tumor necrosis factor alpha (TNF-α) cause sarcopenia (Kim et al. 2021). Inflammatory response induces a reduction on satellite cells production causing degradation of muscle tissue (Kim et al. 2021). Also, inflammatory upregulation in conjunction with the increase of E3 ubiquitin and MuRF1 triggers decrement of ubiquitin–proteasome system (UPS) which is also connected with the degradation of muscle tissue (Kitajima et al. 2020). Furthermore, the involvement of p38 MAPKs which upregulate the p16Iu4a manifests inflammatory increment and satellite cell decrement, leading to sarcopenia (Kim et al. 2021). Fibroblast growth factors 2 and 6 (FGFs 2, 6) increment and decrement, respectively, induced a downregulation of satellite cells causing muscle tissue degradation (Kim et al. 2021). Reactive oxygen species (ROS) upregulation negatively affects mitochondrial function, causing myosteatosis, a condition which increases adipose tissue depots in skeletal muscle (Kim et al. 2021). In addition, mitochondrial downregulation increases autophagy, a catabolic process which could result in sarcopenia, as well (Kim et al. 2021).

The multifactorial pathophysiological pathways of sarcopenia make it difficult to find out which specific mechanism causes this syndrome. Also, the different lifestyle factors augment even more the difficulty to interpretate sarcopenia. Thus, the study of lifestyle factors needs extended research. Lifestyle factors such as: physical activity, nutrition/diet, sleep duration and quality, unhealthy habits or substances (nicotine and alcohol), etc., affect the expression of sarcopenia, reversing or exacerbating the symptoms.

The lifestyle factors which will be further analyzed are:

(i) Physical activity
(ii) Nutrition
(iii) Sleep
(iv) Abuses

Lifestyle Factors

Physical Activity/Exercise

Physical activity contributes, through multifactorial mechanisms, on humans' well-being and quality of life. Research has widely studied the effect of exercise on sarcopenia. Specifically, it is well established the contribution of physical activity on muscle mass hypertrophy, reducing the degradation of muscle mass, which is observed in sarcopenia (Otsuka et al. 2022).

Longitudinal studies have documented the effect of exercise on sarcopenic patients. Secretion or inhibition of different biomarkers affects lean muscle mass. Biomarkers which contribute to the increment or reduction of muscle mass, while are simultaneously affected by exercise, are the following (Table 3):

(a) TNF-α

TNF-α is a biomarker which is included in both exercise and sarcopenia. Thus, the common clue about its action is the secretion of it in inflammatory

Table 3 Secreted biomarkers through exercise and their results on the symptoms of sarcopenia

Authors	Type of exercise	Biomarkers	Result
Morawin et al. (2021)	Aerobic (Tai Chi)	TNF-α + TNFRI – TNFRII + Cas 8 – Cas 9 – cf. DNA =	Reduce sarcopenia symptoms Loss of fat mass Improve PP Changes in apoptotic mechanisms
Lu et al. (2021)	Aerobic (physical activity)	Creatinine – Irisin – CRP – TNF-α GSSG – DHEA-S – Cortisol + C-peptide – Insulin – Leptin – Hemoglobin +	Improve limbs strength Increase ASMI Loss of gait speed
Greiwe et al. (2001)	Resistance exercise	TNF-α protein content – TNF-α mRNA – LPL +	Increased protein synthesis Increased maximal voluntary strength on full body exercises

+: increased; −: decreased; TNF-α: tumor necrosis factor alpha, TNFRI: soluble tumor necrosis factor receptor I, TNFRII: soluble tumor necrosis factor receptor II, Cas 8: caspase 8, Cas 9: caspase 9, cfDNA: cell-free DNA, PP: physical performance, ASMI = appendicular skeletal muscle index, CRP = C-reactive protein, DHEA-S = dehydroepiandrosterone sulfate, GSSG: oxidized glutathione, LPL: lipoprotein kinase

situations that exercise and sarcopenia show in anabolic and catabolic conditions, respectively (Papadimitriou 2021).

(b) Creatinine

Creatinine is an index of muscle degradation especially in exercise conditions. Therefore, its excessive secretion sometimes means high exercise load (Calles-Escandon et al. 1984), whereas, in syndromes like sarcopenia due to muscle tissue impairment, which is observed, the serum creatinine levels drop (Golino et al. 2020).

(c) Irisin

Irisin is a myokine and its potential action, according to the studies on animals, targets on the fact that alters the white adipose tissue (WAT) to brown adipose tissue (BAT) (Li et al. 2021). Its secretion is observed after exercise, thus, act as a treatment in inflammatory conditions (Lu et al. 2021). However, low irisin levels can be a predictor of sarcopenia, especially when coexisting with obesity (Oguz et al. 2021).

(d) CRP

CRP serves as a predictor for inflammatory situations and raises in sarcopenia. Its secretion during exercises varies according to the type of exercise and the gender. Thus, in exercises which contain heavy plyometric moves, like football, CRP does not alter in comparison with control subjects. Also, women

have lower CRP values than men, probably due to lower levels of muscle mass due to lower testosterone levels (Kasapis and Thompson 2005). Thus, exercise lowers CRP levels, decelerating the loss of muscle strength (Schaap et al. 2006).

(e) Oxidized Glutathione (GSSG)

Oxidized glutathione is an indicator of oxidative stress in the cells. Thus, during prolonged exercise, its levels are increased (Seifi-Skishahr et al. 2016). However, on sarcopenia patients, its levels are decreased (Lu et al. 2021).

(f) Cortisol

Cortisol is a hormone which is observed especially in high-intensity prolonged exercise. On sarcopenia patients, the cortisol levels rises, thus, it is important to control the exercises' load during training (Lu et al. 2021).

(g) Insulin

Insulin is a hormone that regulates the use of blood sugar. Exercise has beneficial effect on the sensitivity of insulin. On sarcopenia patients, insulin sensitivity declines. Thus, exercise probably enhances the sensitivity of insulin on sarcopenia patients. However, the mechanisms remain unclear (Cleasby et al. 2016).

(h) Leptin

Leptin is a hormone and its concentration regulates energy balance, thus affecting the body weight. Long-term or high-intensity exercise reduces, 9 h later, the secretion of leptin. On the other hand, short exercise duration does not affect its levels (Kraemer et al. 2002). In sarcopenic patients, the values of leptin are increased (Waters et al. 2008), and this probably provokes the muscle tissue degradation. Thus, in sarcopenia, low-intensity exercise can contribute to leptin level decrement.

Therefore, it is well established that physical activity and systematic exercise contribute to inhibition of sarcopenia symptoms. According to the latest reports, physical activity (aerobic or/and resistance exercise) reduces the secretion of inflammatory biomarkers such as TNF-α, CRP, Cas 8, 9, etc., (Lu et al. 2021) improving muscle mass, muscle strength, and quality of life of the elderly. However, it is also known that for the adaptation of stimuli, through physical activity, are secreted pro-inflammatory biomarkers, as well (Papadimitriou 2021).

Thus, almost the same biomarkers, as in sarcopenia, are secreted in the muscle mass as a response of adaptation to the stimuli of exercise. The pro- and anti-inflammatory cytokines IL-1α, TNF-α, and IL-1 receptor antagonist (IL-1RA), IL-10, and pleiotropic IL-6, respectively, contribute to the muscle hypertrophy (Papadimitriou 2021). Concluding that the depiction of the exact contribution of each mechanism still remains under research. So, the action of some biomarkers seems to be observed in both sarcopenic and exercise conditions, provoking the degradation and reconstruction of muscle mass, respectively.

Nutrition/Diet

There are many changes that occur with aging such as: (a) energy needs progressively decline (25% decrease between the ages of 40 and 70 years old (Nieuwenhuizen et al. 2010); (b) energy intake decreases (Wakimoto and Block

2001); (c) appetite declines; (d) eat slower; and (e) consume smaller meals (Nieuwenhuizen et al. 2010) than when they were younger. These factors in a combination with socioeconomic changes and alterations in smell and taste (Buford et al. 2010) contribute to a nutrient deficiency which is a major risk factor in the development of sarcopenia. Many biomarkers related to nutrition have been investigated and are used in clinical practice to protect, detect, and treat sarcopenia. Biomarkers related to nutrition have been extensively analyzed in ▶ Chap. 50, "Candidate Biomarkers for Sarcopenia and Relationship with Nutrition." Therefore, a brief review of these biomarkers follows below (Table 4).

A significant mechanism underlying sarcopenia is a protein deficiency. Creatinine – an amino acid found mainly in muscles – is essential to estimate creatinine excretion rate (CER), a useful indirect method to measure total body skeletal muscle mass and muscle strength and physical performance (Stam et al. 2019). In healthy individuals, CER ranges between 18 and 21 mg/kg and 21 and 25 mg/kg in women and men, respectively (Heymsfield et al. 2014). CER varies from 4% to 8%, depending on physical activity and diet *(*Kalantari and Bolton 2013*)*. A high-protein diet may elevate daily creatinine excretion (Tangri et al. 2011). Because of its reliability, easy accessibility, and cost-effectiveness, creatinine is a stable biomarker frequently used in clinical practice to estimate muscle mass. Furthermore, one of the structural components of muscle fibers is 3-methylhistidine, which is a valuable biomarker for detecting increased muscle catabolism (Kochlik et al. 2018), as excreted in the urine during muscle degradation. The main problem for measuring plasma 3-MH is the necessary abstinence from meat for 24 h before blood collection, as it is affected by meat or fish consumption (Kochlik et al. 2018).

Insulin-like growth factor 1 is a sensitive marker of the nutritional status (Estívariz and Ziegler 1997). Low energy and/or protein intake is associated with a major reduction in IGF-1 (Fontana et al. 2008). Especially, essential amino acids can play a crucial role in IGF-1 production (Bonjour 2016). A low concentration of IFG-1 is associated with low skeletal muscle mass; thus, it can be a potential risk factor for the development of sarcopenia (Bian et al. 2020).

Vitamin D deficiency was associated with low muscle mass, low physical performance, and sarcopenia (Remelli et al. 2019). Vitamin D has been suggested as a hormone that can stimulate differentiation and proliferation of muscle fibers, especially type IIA. Type IIA fibers are crucial for short-term and high-intensity anaerobic activities, such as sprint or jumping, and reduce the risk of falling (Remelli et al. 2019). As a result, vitamin D status is a crucial biomarker for the early prognosis of hip fractures, early death, and sarcopenia.

The anti-inflammatory capacity of n-3 fatty acids is well documented. Low serum levels of fatty acids are associated with a risk of sarcopenia, because n-3 fatty acids can protect human muscle homeostasis (Jang et al. 2020) via decreasing inflammation and increasing muscle protein synthesis.

Production of ROS and free radicals (FR) may have an essential role in the process of neuromuscular degeneration, leading consequently to the loss of muscle fibers (Kozakowska et al. 2015). Antioxidants taken from diet, like selenium (Chen et al. 2014), carotenoids (Semba et al. 2007), and vitamins C and E (Bjørnsen et al.

Table 4 Secreted biomarkers are affected by nutritional condition and their results on the symptoms of sarcopenia

Authors	Type of study	Biomarkers	Results
Stam et al. (2019)	RCT	Creatinine and CER –	CER positively associated with muscle mass and muscle performance.
Kochlik et al. (2018)	Article	3-MH +	Increased in muscle degradation. Influenced with meat or fish consumption.
Bian et al. (2020)	Cross-sectional study	IFG-1 –	Low concentration associated with reduce muscle mass.
Minamino et al. (2021)	Cross-sectional study	Vitamin D –	Low vitamin D levels correlated with a high prevalence of severe sarcopenia.
Jang et al. (2020)	Article	n-3 fatty acids –	Low serum levels are associated with a risk of sarcopenia.
Chen et al. (2014)	Cross-sectional study	Selenium –	Low selenium concentration is associated with low muscle mass and involved in the development of sarcopenia.
Semba et al. (2007)	Cross-sectional study	Carotenoids –	Decrease levels of carotenoids are associated with low grip strength and low hip strength.
Bjørnsen et al. (2016)	Double-blind, randomized, placebo-controlled trial	Vitamin C and E –	High dosage of vitamins C and E supplementary increasing lean mass.
Nahas et al. (2021)	Cross-sectional study	Uric acid –	Positive association between uric acid and muscle strength.
Zhang et al. (2017)	Systematic review and meta-analysis	Prealbumin – Albumin –	Reduced concentration indicating in subjects with high risk of malnutrition.
Sergi et al. (2006)	Cross-sectional study	Prealbumin – Albumin – RBP – Transferrin –	All visceral proteins except transferrin are significantly lower in underweight group than the normal group.
Yang and Chen (2021)	Cross-sectional study	Leptin +	Increased serum leptin levels were associated with high risk of sarcopenic obesity.

+: increased; −: decreased; CER: creatinine excretion rate, 3-MH: 3-methylhistidine, IFG-1: insulin-like growth factor 1, RBP: retinol-binding protein

2016), have also been associated with sarcopenia, as low concentrations of these nutrients linked to low muscle mass and strength on sarcopenic patients. Furthermore, higher levels of uric acid, which have strong antioxidant properties, were associated with higher handgrip strength (Nahas et al. 2021). In addition, higher levels of uric acid were also positively correlated with higher levels of creatinine, which, as mentioned above, is positively associated with muscle mass, as well (Kurahashi et al. 2013). Although those biomarkers could be used in clinical practice to estimate muscle mass and function, more evidence is needed in order to understand the antioxidants' mechanisms against sarcopenia.

Visceral proteins like prealbumin, albumin, transferrin, and retinol-binding protein (RBP) can, also, be used to evaluate the nutritional status of elders. Although serum prealbumin and albumin levels are commonly used in clinical practice as nutrition markers, some evidence shows that there is no correlation between those serum proteins and dietary intake (Yeh et al. 2018). However, a meta-analysis shows that prealbumin and albumin serum levels were lower in subjects at high risk of malnutrition than those who were at low risk (Zhang et al. 2017). RBP and prealbumin serum levels showed also a higher correlation with FFM than albumin (Shenkin et al. 1996). Nevertheless, RBP is more difficult to measure than prealbumin, so it is not often applicable in clinical practice. On the other hand, transferrin serum levels are unreliable in estimating mild malnutrition and LBM in elders (Sergi et al. 2006). That is confirmed by a cross-sectional study, where the authors found that visceral proteins (prealbumin, albumin, RBP), except transferrin, are useful biomarkers in detecting malnutrition in elderly patients (Sergi et al. 2006). Therefore, data for these visceral serum proteins and their ability to predict nutritional deprivation are not entirely clear.

In sarcopenic obese individuals, it has been observed increased concentration of leptin (Yang and Chen 2021), which is secreted by the increased deposition of fat mass. High leptin levels may imply high levels of inflammatory markers like TNF-α, IL-6, and IL-12 (Tilg and Moschen 2006). As it is well known, these markers stimulate muscle degradation (Roubenoff et al. 1997), causing muscle mass loss and eventually sarcopenia.

Sleep Quality and Duration

The National Sleep Foundation recommends sleep duration to be somewhere between 7 and 8 h (Hirshkowitz et al. 2015). It seems that the duration of sleep is associated with sarcopenia. Both the few hours of sleep and the long hours of sleep were connected to largest risks of sarcopenia (Kwon and Pessin 2013). Sleep timing, sleep duration, and sleep quality consist all parameters that need to be put under consideration when describing "good sleep" (Rubio et al. 2019). Sleep conditions are associated with body composition and mainly with conditions like sarcopenia or sarcopenic obesity (Piovezan et al. 2019). Three hormonal biomarkers whose secretion is associated with sleep quality and duration have been associated with sarcopenia, too (Table 5). These are:

Table 5 Secreted biomarkers are affected by sleep duration and quality condition and their results on the symptoms of sarcopenia

Authors	Type of study	Biomarkers	Results
Yangita et al. (2019)	Article	Cortisol +	Higher levels in sarcopenic group than the non-sarcopenic.
Bian et al. (2020)	Cross-sectional study	GH and IGF-1 –	Lower values associated with reduced muscle mass and sarcopenia.
Auyeung et al. (2015)	Article	Testosterone –	Sleep duration affects testosterone level, muscle mass and function

+**: increased;** −**: decreased;** GH: growth hormone

(a) *Cortisol*

Chronic disruption of the circadian rhythm has been linked to an increase in cortisol serum level (stress hormone), which can have detrimental effects on the musculoskeletal system (Weibel et al. 1995). Chronically elevated cortisol has been linked to muscle catabolism, which in turn conducts a risk factor for sarcopenia (Yanagita et al. 2019).

The hypothalamic-pituitary-adrenal (HPA) axis modulates sleep and is also closely related to stress and anxiety. Poor sleep quality is recognized from the HPA axis as a stress factor and results in an elevated secretion of cortisol (van Dalfsen and Markus 2018). Therefore, elevated plasma cortisol levels (hypercortisolemia) have catabolic effects, leading to proteinolysis and composing a risk for sarcopenia (Brillon et al. 1995). The cortisol/testosterone ratio is often used as an indicator of the hormonal regulation of muscle hypertrophy (Barrett-Connor et al. 2008).

(b) *Endocrine Factors: GH and IGF-1*

GH (growth hormone) and IGF-1 (insulin growth factor 1) are corresponding hormones that stimulate growth of skeletal, muscle, and bone tissues (Bian et al. 2020). Their decreased level would result in the atrophy of these tissues (Le Roith 1997). The production of these anabolic hormones peak during puberty and decline drastically through the following years. Every 10 years from age 20 for a BMI of 25, GH is reduced by 14%. Additionally, GH further decreased by 5.3% for every unit increase of BMI (Iranmanesh et al. 1991). The decline of GH and IGF-1 is commonly referred to as "somatopause."

Poor sleep prevents us from reaching stages of "deep sleep" which are analeptic periods, related to GH secretion (Prinz et al. 1995). The anterior pituitary gland releases GH in a fluctuating manner throughout the day and reaches its peak after we fall asleep. Seventy percent of daily GH secretion takes place once we are in a state of low-wave sleep (Kaiser and Ho 2016). Due to the diurnal nature of GH, IGF-1, which is secreted by the liver in response to GH, would comprise a more accurate biomarker (Ascenzi et al. 2019).

(c) *Testosterone*

Testosterone is well known for its anabolic effect. It enhances muscle strength and physical function, also improves body composition and quality of life in men (Srinivas-Shankar et al. 2010). As mentioned before, the combination of the

increase in cortisol, a catabolic hormone, and the decrease in testosterone, an anabolic hormone, when an individual is sleep-deprived, are closely related to reduced muscle protein synthesis (Saner et al. 2020).

Serum testosterone levels fluctuate during sleep and wake-up call (Axelsson et al. 2005). Sleep fragmentation or a unhealthy sleep patterns compose a factor that leads to testosterone reduction (Cote et al. 2013). It has been observed that (a) sleep restriction (Leproult and Van Cauter 2011), (b) total sleep deprivation (Su et al. 2021), and (c) partial sleep deprivation (Luboshitzky et al. 2001) play a crucial role in testosterone levels, while other studies have shown no or a weak association between restrictive or partial sleep deprivation and testosterone levels (Smith et al. 2019). However, studies note that low testosterone causes less healthy sleep (Barrett-Connor et al. 2008). This evidence suggest that testosterone may make up an effective biomarker for poor sleep, which may increase the risk of sarcopenia.

Substances' Abuse

Alcohol

Attempting to correlate alcohol consumption to sarcopenia can be quite difficult. According to the literature, the ability of a chronic alcoholic to build muscle is impaired; however, no studies present significant evidence to prove that alcohol consumption increases the risk for sarcopenia.

We should take into consideration that most alcoholics are malnourished and inactive, which is probably the primary reason they show decreased muscle mass (Lieber 2003). Chronic alcoholic myopathy also consists a probable cause, as it leads to atrophy of type II muscle fibers (Preedy et al. 2001), mainly types IIA/IIB and IIB, and can decrease muscle mass by 30% (Preedy et al. 2001). A study in animal models showed that alcohol alone does not cause muscle atrophy, but does when the muscles are immobilized (Vargas and Lang 2008).

A plethora of studies has been focused on the possible correlation between chronic alcohol consumption and the decrease of muscle synthesis, showing catabolic processes in preexisting muscle mass, and actual correlation with sarcopenia (Lang et al. 2009; Preedy et al. 2001). Some studies have examined the deregulation of protein synthesis by alcohol, specifically showing that mainly type II muscle fibers are reduced in alcoholics (Preedy et al. 2001). However, there is a dispute in research, and a meta-analysis study showed no correlation between alcohol consumption and sarcopenia (Steffl et al. 2017) (Table 6).

(a) *mTOR Kinase and mRNA Translation*

As we know, alcohol does not seem to destroy preexisting muscle mass. However, it does impair the construction of new muscle mass, which is highly regulated by mTOR protein kinase (mammalian/mechanistic target of rapamycin) (Lang et al. 2009). mTOR kinase belongs to two protein kinase

Table 6 Secreted biomarkers on alcohol consumption and tobacco use and their results on the symptoms of sarcopenia

Authors	Type of Study	Biomarkers	Results
Simon et al. (2017)	Review	mTOR –	Alcohol consumption inhibits S6K1 leading to an impairment in mTORs' ability to regulate mRNA translation and build new muscle.
Casadevalli et al. (2007)	Article	TNF-α and IL-1β –	Higher in patients with COPD than the control group.
Degens et al. (2015)	Review	COHb +	Increased concentration reduce muscle fatigue resistance.
Zong et al. (2019)	Review	MUL1-Akt +	Increased levels induces Akt (protein kinase) and lead to skeletal muscle atrophy.
Carnac et al. (2007)	Review	Myostatin +	Myostatin acts at two points as a negative regulator of muscle mass development: (a) at the level of fibers during embryogenesis and (b) at the level growth in adults

+: increased; −: decreased; mTOR: mammalian target of rapamycin, TNF-α: tumor necrosis factor α, IL-1β: interleukin 1β, COHb: carboxyhemoglobin, MUL1-Akt: mitochondrial E3 ubiquitin protein ligase 1

complexes, mTORC1 and mTORC2 (Frost and Lang 2011). While alcohol intake does not seem to affect the total content of mTORC1, it seems to alter protein regulated pathways within it, leading to impairment of kinase activity and to catalytic procedures (Korzick et al. 2013).

In order for mTORC1 to instigate protein synthesis, two of its underlying layers, ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), need to be phosphorylated (Kimball and Lang 2018). Alcohol consumption inhibits this phosphorylation, leading to an impairment in mTORs' ability to regulate mRNA translation and build new muscle.

Smoking

Addictive behaviors such as smoking and alcohol consumption are intensified in most developed countries, even during adolescence. Interestingly, adolescents who drank alcohol have a triple probability to smoke than those who did not consume alcohol (Papadopoulou et al. 2017). Cigarette smokers have lower BMI but higher central obesity than nonsmokers, indicating that their weight loss is caused by muscle atrophy and not fat loss (Gea et al. 2013). In addition, alcoholics have less muscle type II fibers, while smokers have less type I fibers. The loss of oxidative phenotype seems to cause a shift from type I to type II muscle fibers in smokers (Krüger et al. 2015). The latter is supported by the fact that in monozygotic twins, the smoker twin had less and smaller type I muscle fibers than the nonsmoker one, excluding the effect of genotype, that one may propose on the matter (Larsson and Orlander 1984).

(a) *TNF-α, IL-1β, and IL-6*

Smoking causes an increase in circulating inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 (Casadevall et al. 2007). These pro-inflammatory constituents lead to protein lysis (Degens 2010). Chronic elevation of these cytokines (especially TNF-α) appears in situations of oxidative stress, which is observed in the respiratory muscles of COPD patients (Barreiro et al. 2005). The upregulation of these genes in patients with COPD leads to a reduction in muscle mass and strength (Casadevall et al. 2007).

(b) *Carboxyhemoglobin (COHb)*

COHb in the bloodstream of smokers can reduce oxygen delivery to the muscles. Carboxyhemoglobin is an indicator of this impairment. The increase of COHb could be compared with a hypoxemic condition. The formation of COHb leads to a reduced oxygen-carrying capacity of the blood and may diminish muscle fatigue resistance (Degens et al. 2015).

Additionally, dysfunctional mitochondria are notorious producers of reactive oxygen species (ROS), which can cause oxidative modifications of skeletal muscle proteins (Rom et al. 2012). ROS: (a) alters myofilament function, diminishing force production and (b) enhances the expression of proteins in the ubiquitin-proteasome pathway, leading to degradation and loss of contractile proteins (Reid and Li 2001). Also, mitochondrial ROS are key regulators of the TNF-α/NF-κB pathway and leads to TNF-α-induced protein loss, which has been analyzed above (Reid and Li 2001).

(c) *MUL1-Akt*

MAFbx and muscle RING-finger protein-1 (MuRF1) belong to the proteasome pathway (UPP), the main pathway that controls intracellular protein degradation in skeletal muscle. Increased gene expression of these E3 ligases have catalytic effects on muscle mass and thus are considered "atrogenes" (Foletta et al. 2011).

Specifically on cigarette smokers, MUL1 (mitochondrial E3 ubiquitin protein ligase) induce Akt (protein kinase) reduction and cause degradation, endothelial cell death, and dysfunction (Kim et al. 2016). MUL1 is a novel Akt ubiquitin E3 ligase. Cigarette smoking can noticeably increase MUL1 expression and Akt ubiquitination, and lead to skeletal muscle atrophy (Zong et al. 2019).

(d) *Myostatin*

Smoking increases the myostatin expression, a deactivator of protein kinase B (Akt), a protein that induces protein synthesis (Jackman and Kandarian 2004). Similar to MUL1, which has been explained above, myostatin is considered a muscle growth inhibitor.

Management of Sarcopenia

Physical Activity/Exercise

Aerobic physical activity and resistance training are well established for their therapeutical contribution on diseases and syndromes. World Health Organization (WHO) characterizes physical activity as a prescription for humans' well-being,

physical and mental health (Izquierdo et al. 2021; WHO 2002). Quotes about "Exercise as medicine" indicate the importance of physical activity. Structured exercise should, therefore, be determined on the grounds of the intended effect (e.g., primary prevention, improvement of physical fitness, functional status or therapy of the disease), and should be personalized, and be regulated in the exact same manner as any other form of medical treatment.

Patients who participate on physical activity programs, following a balanced diet combined with moderate alcohol consumption and no tobacco use, can effectively increase their quality of life. On the contrary, sedentary behavior and inadequate exercise are significant risk factors for all-cause and cardiovascular mortality, obesity, sarcopenia, frailty, and disability, along with other chronic aging-related diseases (Izquierdo et al. 2021).

Physical activity has multiple beneficial effects:

- Physical function (Izquierdo et al. 2021)
- Quality of life (Izquierdo et al. 2021)
- Management of frailty (Izquierdo et al. 2021)
- Physical performance (Wu et al. 2021)
- Muscle strength (Wu et al. 2021)
- Mitochondrial biogenesis (Harper et al. 2021)

In elders, an training program performed two to three times per week, for 3–5 months of 150 or 75 min of low or vigorous (85–95% peak heart rate), intensity aerobic exercise, respectively, with 1–4 min rest, combined or not with resistance exercise can be utilized declining sarcopenia symptoms (Izquierdo et al. 2021). Resistance exercise is widely known about its vigorous benefits on sarcopenia symptoms. The latest aspects of literature provide that high load or progressive resistance training inhibits sarcopenia and raises aerobic capacity, respectively (Izquierdo et al. 2021).

Also, Beckwée et al. (2019) reported that high-intensity resistance training program is the preferred option for sarcopenic patients, whereas low-intensity resistance training can be sufficient to induce an increase in strength. Thus, they recommend the following training pattern: 1–4 sets of 8–15 repetitions during 2–3 training sessions per week. Also, low to moderate intensity resistance exercise (40–60% of 1 RM) is as beneficial, for the decrement of sarcopenia symptoms, as the vigorous intensities ($\leq$ 80%) (Otsuka et al. 2022).

Concluding, aerobic, resistance, whole-body vibration therapy and multimodal exercise programs have been recommended for patients' improvement (Papadopoulou 2020a). Specifically, a multicomponent training program consisting of resistance, endurance, gait, and balance exercises should be implemented in order to avoid falls, fractures, institutionalization, disability, frailty, and sarcopenia (Izquierdo et al. 2021).

In addition, resistance exercise combined with other lifestyle factors enhances its effectiveness on deal with sarcopenia. Specifically, nutrition such as food and supplements have been proved by many authors, that their combination with resistance exercise can decelerate the reduction of muscle mass (Wu et al. 2021).

Nutrition/Diet

Diet plays a key role in the prevention and treatment of sarcopenia, although dietary interventions have not been studied extensively. The mechanisms that are involved in sarcopenia do not fully interpret, but they all seem to affect the balance between protein synthesis and degradation rate which subsequently lead to loss of muscle mass. As already mentioned, nutrients that have been strongly associated with sarcopenia are (a) proteins and amino acids, (b) vitamin D (Kaiser et al. 2010), (c) omega 3 fatty acids (Smith et al. 2011), and (d) antioxidants, like vitamin C and E, selenium, and polyphenols (Kaiser et al. 2010).

Protein and Amino Acids

Protein intake provides amino acids, which are required for muscle synthesis. Also, there is a general concern that elders display a blunted muscle production, raising the possibility that overall recommendation for protein intake should be higher for the elders. So, why does the elderly need more protein? The reasons are: (a) inadequate dietary protein intake (loss of appetite, gastrointestinal problems, reduced energy intake, changes in dietary preferences), (b) reduction in utilization of available protein (postprandial protein synthesis, insulin resistance, higher intestinal and liver utilization) (Wall et al. 2015), (c) higher basic energy needs (acute and chronic diseases, inflammatory diseases, increased protein oxidation) (Reidy and Rasmussen 2016), and (d) a higher amount of protein (25–30 g) is required for the threshold of maximum activation of muscle protein synthesis (Martone et al. 2017).

The time of protein consumption is also important for muscle synthesis, given each meal allows protein accretion for 2–4 h. Increasing meal frequency and providing adequate and evenly distributed protein intake between meals result in maximal protein anabolism via postprandial protein accumulation. When protein consumption is evenly distributed between meals, the 24-h muscle synthesis rate is 25% higher in healthy men and women, compared to isoenergetic and iso-nitrogenous diets with an uneven allocation of protein across meals. Protein-rich drinks, consumed about 2 h after dinner and 30 min before bedtime, contribute to muscle protein synthesis, muscle recovery, and overall metabolism (Baum and Wolfe 2015). Considering the above, it can be concluded that depending on the health status, maintaining protein intake while handling the amount and quantity of protein in each meal is essential.

Leucine, an essential amino acid, has been shown to trigger protein synthesis pathways (Zengin et al. 2018). The richest sources of leucine are lean meat, whey products, dairy products, peanuts, lentils, and black beans (Rondanelli et al. 2021). Whey protein contains a high percentage of leucine that provides an essential amino acid mixture, necessary to reverse suboptimal protein synthesis. Co-ingesting 2.5 g of crystalline leucine with pure dietary protein can boost anabolic response in older men (Wall et al. 2013). Furthermore, lower values of skeletal muscle index, grip strength, and physical performance were associated with lower blood leucine levels

(Rondanelli et al. 2021). Whey protein appeared to be more effective in postprandial muscle protein accumulation than casein and casein hydrolysis, due to its (a) kinetic absorption, (b) faster digestion, and (c) higher amino acid concentration and lower nitrogen urine excretion especially leucine (Papadopoulou 2020a; Phillips and Van Loon 2011). Also, casein is digested slowly and progressively during sleep (Res et al. 2012). According to ESPEN guidelines, an intake of 3 g of leucine at three main meals along with 25–30 g of protein is essential to prevent lean mass loss in elders (Rondanelli et al. 2021). In addition, the intake of 30–40 g of casein (a dairy protein) 30 min before bedtime or via nasogastric tube increased overnight muscle protein synthesis in both young and older men (Kerksick et al. 2017). Dairy foods are a good source of high-quality protein and contain various essential nutrients. An observational study found a positive association between dairy consumption and both appendicular bone mineralization and muscle mass in older women. Also, dairy protein and calcium intake are positively correlated with bone density (Papadopoulou et al. 2021; Radavelli-Bagatini et al. 2014).

In conclusion, in elders, protein intake ranging from 1–1.2 g/kg body to 2.5 g weight/daily is necessary to enhance muscle protein synthesis (Wall et al. 2015). These amounts of protein intake should be equally distributed throughout the day, 4–6 times, every 3–4 h, in the total of 20–35 g, containing high levels of leucine (2.5–3 g) (Papadopoulou 2020a, 2020b; Papadopoulou et al. 2021; Wall et al. 2015).

Vitamins and Antioxidants

Vitamin D has been linked with the pathogenesis of sarcopenia and other disease (Papadopoulou 2020a). Deficiency in vitamin D is related to skeletal mass reduction and frailty. Additionally, vitamin D polymorphisms have been linked to muscular strength (Papadopoulou 2020a). A systematic review and meta-analysis of several populations indicated that 400–4000 IU/day of vitamin D supplementation for 1–60 months improved muscle strength, in elders above 65 years old (Beaudart et al. 2014). Few foods are natural sources of vitamin D, like fatty fish (sardines, salmon, tuna, and fish liver oils), and smaller amounts are found in eggs and red meat. Also, certain foods, such as margarine, and breakfast cereals, are fortified with vitamin D (Papadopoulou 2020a). The InCHIANTI cohort study found that carotene, vitamin E, and vitamin C were positively associated with skeletal muscle mass and overall physical performance (Cesari et al. 2012). The antioxidant β-carotene, mainly, protects against the natural decreasing tend of in walking speed, during aging (Lauretani et al. 2008). Inadequate consumption of fruits and vegetables may defend against oxidative stress and decrease the risk of sarcopenia.

The effect of *vitamin* B12 deficiency on both frailty and sarcopenia has been extensively investigated (Behrouzi et al. 2020). Reductions in lean body mass, total skeletal mass, and skeletal muscle mass index were observed in patients with low vitamin B12 levels (less than 400 pg/mL), compared to patients with higher levels of vitamin B12 levels (above than 400 pg/mL). Furthermore, patients with sarcopenia had low B12 levels (lower than 400 pg/mL), indicating a relationship between

vitamin B12 levels and the development of sarcopenia (Ates Bulut et al. 2017). Vitamin B12 intake was related to higher scores in chair stand test, but not with higher SPPB scores (Behrouzi et al. 2020). Therefore, B12 vitamin has a protective effect against the development of sarcopenia, but more research is needed.

Omega-3 fatty acids have a protective role in fighting sarcopenia. An 8-week randomized controlled study in elders found that the group supplemented with omega-3 had a higher rate of muscle protein synthesis than the control. Fatty fish consists of good source of *omega-3 fatty acids*. Grip strength is associated with fish consumption. More specifically, grip strength improved by 0.48 kg in females and 0.43 kg in males for every portion of fatty fish consumed (Smith et al. 2011).

In addition, in elders aged 65–80 years, resveratrol combined with exercise is associated with an increased mitochondrial density and a reduced resistance to muscle fatigue. Furthermore, resveratrol could better protect against sarcopenia compared to exercise alone, by increasing muscle fiber size and strength. The combination of dietary interventions and exercise may produce maximum effects against sarcopenia (Papadopoulou 2020a).

Sleep

Except for monitoring the above factors, planning a healthy sleep schedule and a better bedtime routine (shower, pajamas, etc.) conditions can be created that will prevent the occurrence of sarcopenia can be a strong tool in preventing the appearance of sarcopenia. As stated previously, "a healthy sleep schedule" combines many factors: duration of sleep (7–8 h), sleep timing (aligned to the circadian rhythm), and quality of sleep (time in slow wave sleep, absence of sleep apnea). Wearable sensors are becoming more and more popular, allowing people to monitor their sleep patterns, observe them and change them when necessary.

Interestingly, sensor gives the opportunity for the individual to observe how much time was spent in deep wave sleep, which is the highest quality rest time. Studies on the effects of sleep on sarcopenia differ as to what duration is considered adequate, poor, or good, and what is defined as sleep quality. For example, they defined 6–8 h/sleep as insufficient and $\geq$8 h/sleep as sufficient and showed that people who slept 6–8 h had a higher prevalence of sarcopenia (Rubio et al. 2019). In the KNHANES study (Kwon et al. 2017), the prevalence of sarcopenia was slightly higher in the 8-h sleep group, in comparison with the 7-h group (15.9% and 13.1%, respectively), which could, however, be considered not significant. This leads us back to the National Sleep Foundation's original statement that the best sleep duration is probably between 7 and 8 h, but it probably varies from individual to individual.

Smoking and Alcohol

Smoking and alcohol consumption are associated with chronic diseases, like COPD and liver disease, which can develop metabolic damage causing muscle mass function

to decrease (Prokopidis and Witard 2021), leading to sarcopenia. According to the CDC, smoking is one of the first causes of death and costs more than $225 billion in medical care for adults (CDC 2021a). Cessation of smoking is one of the most important actions as it can: (a) improve overall health status and e quality of life, (b) reduce the risk of cardiovascular disease, and (c) reduce the risk of many other diseases such as lung cancer and other types of cancer, chronic obstructive pulmonary disease (CDC 2021b), and sarcopenia (Steffl et al. 2017). On the other hand, alcohol consumption is associated with both negative (e.g., psychiatric disorders, cancer, liver disease) (Rehm 2011) and positive (e.g., higher self-esteem and optimism, reduced loneliness) (Hajek et al. 2017) health-related outcomes. However, any potential benefits of alcohol are small and may not apply to everyone. The recent Dietary Guidelines for Americans (USDA 2020) recommend clearly that no one should start drinking. Furthermore, American Guidelines point out that alcohol consumers should limit alcohol to two drinks or less per day for men and to one drink or less for women per day in order to reduce the risks of alcohol-related health issues development.

Conclusion

Modifiable behavioral factors such as physical activity, dietary habits, smoking cessation, alcohol moderation, sufficient and quality sleep are associated with sarcopenia and may play a crucial role in prevention of its symptoms. Specifically, a multicomponent training program consisting of resistance, endurance, gait, and balance exercises should be implemented in order to avoid falls, fractures, institutionalization, functional disability, frailty, and sarcopenia Moreover, a balanced diet with adequate protein consumption, vitamins, and minerals can, also, increase muscle tissue mass and prevent the degradation. Furthermore, substance abuses like alcohol and tobacco can affect the symptoms of sarcopenia. Thus, alcohol consumption should be limited to two drinks or less per day for men and one drink or less for women, whereas tobacco should be quit. In addition, adequate and quality sleep can effectively affect the syndrome's remission. So, further research is needed.

Applications to Prognosis, Other Diseases or Conditions

Lifestyle factors such as physical activity or exercise, healthy eating habits, the avoidance of abuses, and sleep quality are prerequisite to cope with sarcopenia. Therefore, a multicomponent training program consisting of resistance, endurance, gait, and balance exercises should be implemented in order to avoid falls, fractures, institutionalization, disability, frailty, and sarcopenia. Also, they should follow a balanced diet avoiding abuses like smoking and reducing alcohol consumption. Last but not least, the quality of sleep can be a strong tool in preventing sarcopenia. Moreover, as stated, a sleep duration between 7 and 8 h can positively affect the syndrome's remission. Further research is needed the effect of certain lifestyle factors on sarcopenia.

Key Facts of Physical Activity Based on World Health Organization (2020)

- Physical activity is beneficial for cardiovascular, body, and mind function.
- Physical activity manages diseases such as cardiovascular diseases, cancer, and diabetes.
- Physical activity inhibits depression and anxiety.
- Physical activity enhances mental skills.
- Globally, 1 in 4 adults prefer sedentary lifestyle.
- Up to five million deaths a year could be avoided if the population was participated in physical activity programs.
- People who are inactive have a 20–30% increased risk of death compared to people who participate in a physical activity program.
- More than 80% of the world's adolescent population has a rare participation in physical activity programs.

Mini Dictionary of Terms

Aerobic exercise is typically moderate-intensity exercise performed for prolonged periods to improve cardiovascular function depending on aerobic energy production and refers to the use of oxygen to adequately meet energy requirements during exercise through aerobic metabolism. In aerobic exercise, oxygen is used for the oxidation of glucose and fats to produce adenosine triphosphate, the main energy carrier for all cells. Aerobic exercise involves regular movements of parts of the body, like legs and arms that increase the workload on the cardiovascular system. There are different types of aerobic exercise. Some examples are walking, cycling, long-distance running, etc. Regardless of the exercise type, it is necessary to maintain an adequate aerobic dose of 40–60% of maximum aerobic capacity (Vo2 max).

Resistance exercise is any exercise that causes the muscles to contract against an external resistance, targeting on increases in strength, tone, mass, and/or endurance. Resistance training works by causing microscopic damage to the muscle cells (catabolism), which in turn are quickly repaired by the body to help the muscles regenerate and get stronger (anabolism). The external resistance can be dumbbells, rubber exercise tubing, your body weight, resistance bands, bricks, bottles of water, or any other object that causes the muscles to contract.

Antioxidants are chemicals that prevent or repair cellular damage caused by exposure to oxidizing agents, such as oxygen, as well as other oxidizing agents produced by the body itself. Several different antioxidants are found in food, while others are produced by the body itself.

Inflammation is the reaction of the body's immune system to an irritant. The irritant can be a microbe or a foreign object. An inflammation starts when the body

tries to fight the harmful irritant. Many different immune system cells may be involved in an inflammatory reaction. These cells release various substances, known as inflammatory mediators, which (a) dilate the small blood vessels allowing more blood to reach the injured tissue and (b) make it easier for immune cells to pass outside the small blood vessels allowing more of them to enter the affected tissue. In some diseases, the immune system mistakenly fights the body's cells, causing harmful inflammation.

Lean body mass are all body tissues other than fat, namely muscles and minerals (such as those found in bones and water).

Muscle atrophy is due to decreased muscle protein synthesis and protein degradation caused by various pathophysiological conditions such as disuse, aging, cachexia, cancer, diabetes, obesity, and others.

Muscle degradation can take place either intracellularly or extracellularly. During food digestion, digestive enzymes are released for extracellular digestion, where proteolytic cleavage breaks down proteins into smaller peptides and amino acids to allow them to be absorbed and used.

Physical function is the ability to perform basic activities of daily living, like dressing, eating, bathing, etc. There are several physical function tests where used in clinical practice (SPPB, TUG, 400m walk). In one of the three criteria for diagnosis sarcopenia, based on EWGSOP 2 and SDOC criteria. It consists one of the three criteria for diagnosis sarcopenia.

Free radicals are molecules that are missing electrons. The absence of electrons causes this chemical to react and oxidize other molecules, taking electrons from them. Free radicals can damage lipids, cell membranes, DNA, and tissues, altering their chemical structure and function. On the other hand, they are produced as a normal cellular metabolism.

Summary Points

- There is no specific mechanism that provokes this syndrome.
- The different lifestyle factors and ways in which people live nowadays augment the difficulty to interpretate sarcopenia.
- Multiple secretion of biomarkers affects the expression of the syndrome positively or negatively.
- Lifestyle factors such as physical activity, nutrition, sleep, and abuses can affect the secretion of biomarkers.
- Tumor necrosis factor, interleukins, and hormones can affect positively or negatively syndrome's symptoms.
- The management of sarcopenia necessitates the contribution of various lifestyle factors.
- Patients who participate in physical activity programs, adopt healthy nutrition habits, and avoid abuses can effectively cope with this syndrome.

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Body Composition, Basal Metabolic Rate, Resting Energy Expenditure, and Other Surrogate Measures as Biomarkers in Nutrition: Applications to Anorexia Nervosa

53

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Abstract

Anorexia nervosa (AN) is a complex disorder of unknown etiology. It is characterized by dietary restriction and severe malnutrition, as well as by many psychological disturbances. The awareness of AN has risen in the last decades among scientists of different disciplines. It is undeniable that AN has the highest mortality rate of all mental disorders. There is no doubt that AN has the highest mortality rate of all mental disorders. In the treatment of this disorder, nutritional recovery and weight restoration are premature goals. It seems that nutritional status influences both the effectiveness of an intervention's programs and the general health of this population, and it is often the result of interrelated factors. Experts should consider that while weight restoration is a critical part of AN treatment, changes in body composition, energy, and nutrient needs during weight recovery as well as weight maintenance should also be taken into consideration. This chapter attempts to describe suggested biomarkers related to energy requirements as a means of selecting the most appropriate and reliable methods of nutritional assessment.

Keywords

Anorexia nervosa · Biomarkers · Nutritional assessment · DEXA · BIA · Equations

Abbreviations

%BF	Body fat percentage
2C	Two-compartment model
3C	Three-compartment model
4C	Four-compartment model
AN	Anorexia nervosa
APA	American Psychiatric Association
BC	Body composition

BIA	Bioelectrical Impedance Analysis or Bioimpedance Analysis
BMR	Basal metabolic rate
CSA	Cross-sectional area
CT	Computed tomography
DR	24-hour dietary recall
DXA	Dual-energy X-ray absorptiometry
ED	Eating disorders
FAO	Food and Agriculture Organization of the United Nations
FFM	Fat-free mass
FFQ	Food frequency questionnaire
FM	Fat mass
Pha	Phase angle
REE	Resting energy expenditure
SAT	Subcutaneous adipose tissue
ST	Skinfold thicknesses
TBW	Total body water
VAT	Visceral adipose tissue
WC	Waist circumference
WHtR	Waist-to-height ratio

Introduction

Eating Disorders

Eating disorders (ED) are characterized by a persistent disturbance of eating or eating-related behavior that results in the altered consumption or absorption of food and that significantly impairs physical health or psychosocial functioning (DSM IV). They are often very private and hidden issues, which can exist for a long time before they are identified. ED affect people unrelatedly of gender, age, and ethnicity. Regardless of the complexity of combining all ED prevalence data, the most recent studies confirm that ED are highly prevalent worldwide, especially in women (Schmidt et al. 2016; Galmiche et al. 2019). Merikangas et al. (2010) study showed that ED are more prevalent among young women (3.8%) than among young men (1.5%) in the USA. One quarter of the participants in the study mentioned above were males with anorexia nervosa (AN). Men have an increased risk of dying because they are diagnosed much later than women. This trend could be in part due to the misconception that men do not experience eating disorders (Disorders Resource Catalogue 2014). According to Galmiche et al. (2019), global eating disorder prevalence increased from 3.4% to 7.8% between the years 2000 and 2018. National Eating Disorders Association refers that 70 million people internationally live with ED. Japan has the highest prevalence of eating disorders in Asia, followed by Hong Kong, Singapore, Taiwan, and South Korea (Pike and Dunne 2015) and Austria had the highest rate of prevalence in Europe at 1.55% (Psychology Today 2013). It is important to mention here there is a claim that lockdown measures associated with the COVID-19 pandemic are having adverse consequences

for people's mental health, including increases in maladaptive eating habits and body dissatisfaction. Eating disorders (EDs) are no exception, with incidence and prevalence of ED rising since COVID-19 starting (Schlegl et al. 2020).

There is an increasing interest in factors contributing to the etiology of ED because there is no distinct cause of these abnormal eating behaviors. There are theories which argue that it is the result of several factors such as genetic, biological, behavioral, psychological, and social (American Psychiatric Association 2013). Research indicates that neurophysiological and neuropsychological factors play an important role in the pathogenesis of eating disorders (Mishra et al. 2017).

Treatment of ED is a complex and interdisciplinary endeavor. Because of the effect of eating disorders on physical and mental health, treatment usually involves psychological, pharmacological, and nutritional counseling and surveillance. The act or manner of treating ED needs to be adjusted to these serious disorders and should take in mind the physical aspects of these abnormal eating behaviors (clinical consensus point). Therapy of ED is a long process that will continue for months or years even if all goes well. It can also be differentiated into outpatient, hospital, or day care patients. With young patients (children and adolescents) who are still living with their family, the parents, close relatives, or guardians should be involved in the treatment (American Psychiatric Association 2013; National Collaborating Centre for Mental Health 2004). It is noteworthy that the dropout rate of the treatment of ED is very high, ranging from 20.2–51% for inpatients and from 29–73% for outpatients (Fassino et al. 2009).

Anorexia Nervosa

The history of AN starts with descriptions of religious fasting dating from the Hellenistic era and continuing into the medieval period. The English physician Richard Morton, in 1689, describes his concern with anorexic behaviors of patients. The term *anorexia nervosa* was first used by Gull (1873) to describe a self-starvation syndrome. In the same year, Lasègue, similarly, published details of a number of cases in a paper entitled *De l'Anorexie Hystérique* (Lasègue 1873).

There is evidence that anorexia nervosa (AN) is an ED which has the most serious consequences in a person's life with organic, behavioral, psychological, and social consequences. It is characterized as a severe chronic mental disorder with a 12-month prevalence rate of 0.4% among females, and approximately one-tenth of that among males (American Psychiatric Association 2013). The prevalence of AN is reportedly between 1–4% of young women and 0.3–0.7% of young males in Europe (Keski-Rahkonen and Mustelin 2016). It also has the highest mortality rate of any psychiatric disorder and is more than five times higher than the mortality in the general population matched for age and sex (Edakubo and Fushimi 2020).

The exact pathogenesis of AN remains uncertain and still unknown, as AN is a multifactional disorder. Several explaining proposals include genetics and biology, as well as psychological and emotional health causes. Family, history, other mental health disorders, dieting and starvation, culture-related diagnostic issues, and stress may rise the danger of developing AN (Bulik et al. 2016).

There are two subtypes of AN: restricting type and binge eating or purging type. The American Psychiatric Association (2013) suggests that subtype description should be used to describe current symptoms rather than longitudinal course (DSM V). Individuals with AN are characterized primarily by abnormally low energy and nutrient intake and low body weight, typically displacing an intense fear of gaining weight or of becoming fat. Their self-esteem, also, is extremely dependent on their perceptions of body shape, body image, and weight. A subgroup of individuals with AN exhibits an excessive level of physical activity. So, the risks associated with AN, among others, are malnutrition, dehydration, electrolyte imbalances, muscle weakness, refeeding syndrome, gastrointestinal problems, slowed or irregular heartbeat, weakness and fatigue, body dysmorphia, and loss or disturbance of menstrual periods in girls and women.

The treatment of AN is a long process and is somewhat different for children, adolescents, and adults. Commonly, it involves a combination of therapies, medical sequelae, combined with psychiatric, dietary specialization, and monitored weight gain. A major issue for an individual with AN is to get treatment as soon as possible, to reduce the risk of serious complications, especially if the anorexic has lost a lot of body weight (American Psychiatric Association 2006; National Guideline Alliance [UK] 2017).

Nutritional Counseling of Anorexia Nervosa

Initial focus of a nutrition therapy, regardless of age, gender, and perhaps the period of the disease, is to safely increase energy intake and stabilize weight loss to begin nutrition rehabilitation in order to restore weight, eliminate binge eating/purging behaviors, and other ritualistic eating patterns (Pettersson et al. 2021). Hence, it is very important before any nutritional approach to evaluate, as correctly as possible, caloric needs and energy expenditure, in order to enable clinical nutritionists to identify the clinical picture and treatment progress and to configure eating plans according to personal needs (National Guideline Alliance [UK] 2017). Additionally, it is essential to mention that the energy needs of anorexics differentiate according to the rehabilitation phase or refeeding stage (e.g., starvation and weight-restored phase). Moreover, it is crucial to keep in mind to avoid refeeding syndrome and underfeeding of anorexics (Tresley and Sheean 2008). Finally, it is significant to note that excessive physical activity is one of the most paradoxical features of anorexia nervosa (Melissa et al. 2020).

Body Composition and Anthropometric Measurements

Although direct and indirect calorimetry represents the most accurate technique to evaluate energy balance in patients, measurement of body composition is considered as a surrogate measure of metabolically active tissues (Agüera et al. 2015). Additionally, the restoring body fat mass in the treatment of AN is vital since normal level of body fat percentage (%BF) is necessary for regular menses in women of reproductive age; it is essential for health, such as the return of menstruation and bone

health (Ayton 2019). Furthermore, failure to achieve a normal body composition is a risk factor for relapse (Bodell and Mayer 2011).

Body composition (BC) represents dietary intakes, losses, and expenses during a long period. It is widely accepted that measuring BC we collect data and information to evaluate the nutritional status in hospitalized patients and in outpatients with nutritional risks as malnutrition. So, it is essential for designing nutritional strategies and monitoring of nutritional interventions during nutritional rehabilitation (Agüera et al. 2015; Andreoli et al. 2016). Studies demonstrated alterations in body composition traits in patients with AN before and after treatment (Hübel et al. 2019).

Several techniques for BC measurement are available in clinical population. The anthropometry, body weight, body mass index, abdominal circumference (those are noninvasive and do not require the use of electronic devices), and bioelectrical impedance analysis are the most used indirect methods for the assessment of body fat percentage because these methods are simple to perform and low in cost. Dual-energy X-ray absorptiometry (DEXA), computerized tomography (CT), total body water, hydrostatic weighing, nuclear magnetic resonance, and computed tomography and magnetic resonance are characterized as direct and more accurate measures of body fatness (Lemos and Gallagher 2017). The body composition can be analyzed on the anatomical or the molecular level.

Although the two-compartment (2C) model divides the body weight into fat-free mass and fat mass and uses assumptions that ignore interindividual variability in the composition of FFM, it is the most widely used model to estimate body composition in general population. Using this model requires that body hydration level and bone mineral content are stable (Toomey et al. 2015). Several studies show that the 2C model cannot safely be applied to AN patients without running the potential risk of having large errors in estimation of components of body composition (Peterson et al. 2003; Toomey et al. 2015).

The three-compartment (3C) model divides body weight into fat, water, and remaining fat-free dry tissue, and requires measurements of body weight, body water by hydrometry, and body volume by densitometry (differentiating TBW + FM + fat-free dry tissue). The fat-free mass can be separated into water, mineral, and protein. In a multicomponent model, the fat-free mass varies by age, sex, and race, as each subcomponent in the fat-free mass changes complies with specific situations (Toomey et al. 2015).

The most accurate method for determining body fat on the molecular level is the four- compartment model (4C). This model divides body weight into fat mass, bone mineral density, water, and proteins and determines the total body fat content. Combining several measurement techniques to divide body mass into fat (measured by hydrodensitometry), mineral (measured by DXA), water (measured by isotope dilution), and protein (residual) (Lara et al. 2014), the 4C model estimates BC accurately.

Many methods of measuring body composition are validated and feasible in AN. Meta-analyses stated that the pretreatment body composition of individuals with AN is significantly altered (Hübel et al. 2019). Many studies exhibited that among patients with AN body composition may vary depending on many factors

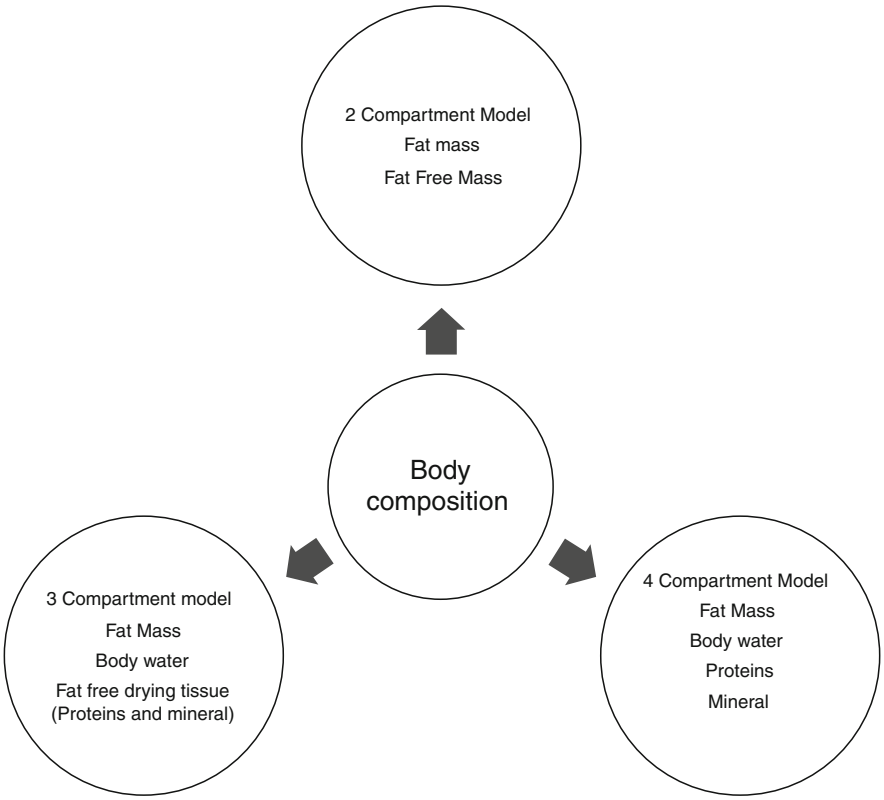

Fig. 1 Models of body composition

like age, gender, phase of treatments, medical condition, stage of refeeding and maturity level, level of physical activity or excessive exercise, eating habits/diet, bone mass density, vomiting, laxative use, ethic differences, fluid, and age of the onset of the disorder (Mattar et al. 2011a; Probst and Goris 2012; Fernandez-del-Valle et al. 2015; El Ghoch et al. 2017; Nagata et al. 2017; Nagata et al. 2019) (Fig. 1).

Bioelectrical Impedance Analysis or Bioimpedance Analysis (BIA)

Bioelectrical impedance analysis or bioimpedance analysis (BIA) estimates fat-free mass using a two-compartment model and mathematical equations. It is based on the measurement of the electrical resistance of the body or a body region that can be quantitatively related to the amount of water in the tissues. BIA involves the crossing of a minor electrical current through the body and measuring the resistance offered. Many prediction equations have been developed for specific populations including obese, adults, elderly, and children. FM is calculated indirectly by subtraction of the

FFM from the total body weight. It is a noninvasive, low-cost technique and does not require operator training. BIA is a reliable technique in healthy and clinical population when hydration status is constant (stable fluid/electrolytes balance) and using the proper equation (Piccoli et al 2005; Mattar et al. 2011b).

Recently, numerous frequencies BIA has been developed which allows prediction independently of hydration status, in men and women with a wide range of body mass index. While Achamrah et al (2018a, b), in a retrospective study on 3655 measurements in patients with BMI between 16 and 18, suggest that BIA and DXA methods are interchangeable at a population level, but at the individual level it is lacking and studies are needed to develop new BIA-specific equations according to the BMI class. Similarly, Abbaspour et al. (2021), using foot-to-foot BIA, compared DEXA and BIA in the assessment of FFM, FM, and body fat percentage in women with AN upon admission and discharge, and suggest the development of more specific BIA equations in order to improve validity and precision of BIA in patients with AN.

Although BIA is simpler and quicker to use than other techniques and the number of AN patients examined is large, the peculiarity of the disorder requires further studies to confirm the reliability of the method, which remains questionable in severely malnourished patients in individual level (Mattar et al. 2011b; Achamrah et al. 2018a, b). Studies proposed that the use of BIA at 50 kHz is not superior to Wt for assessing total and leg FFM and may be useful in assessing body composition in malnourished anorexic patients, as compared to Wt alone, because it allows a more appropriate evaluation of intracellular water.

With respect, BIA may be more accurate for patients with AN-successive clinical renourishment (normal BMI, FFM, FM, and hydration status) using the equations developed in healthy populations (Mattar et al. 2011b; Coëffier et al. 2020; Abbaspour et al. 2021).

Phase Angle (PhA)

Phase angle (PhA) is calculated based on resistance (R) and reactance (Xc) obtained by means of bioelectric impedance analysis according to phase angle formula PA = arc tangent Xc/R. The phase angle is an important parameter for estimating an organism's health status as well as the cellular health (Małecka-Massalska et al. 2017). PA is commonly accepted as a prognostic indicator of morbidity and mortality. The Pha can be used to evaluate the extracellular/intracellular water distribution in clinical population and is an accurate indicator of qualitative changes in body composition. Studies in anorexic population show a relationship between PhA, nutritional status, and an objective and useful indicator of correct nutritional status in clinical practice (Lukaski et al. 2017). It is also a significant predictor of BMR even if considered together with weight and age. A low PhA ($< 5°$) is a common finding in severe malnutrition, the higher phase angle value is correlated with better cell function and more imperishable the cell membranes are (Małecka-Massalska et al. 2017; Speranza et al. 2021).

Dual-Energy X-Ray Absorptiometry (DXA)

DXA was initially developed for the measurement of bone density with the purpose of ensuring the early diagnosis and monitoring of osteoporosis. DXA now also applies to determining the composition of soft tissues. Several types of DXA devices are available, but they all operate in the same manner. It is a 3C chemical model of body composition, a noninvasive and direct assessment, capable of providing estimates for three separate components of BC, the FM, FFM (water, protein, glycogen, and soft tissue minerals), and bone mineral density. It is often described as a gold standard and is widely available, but requires specialized radiology equipment and an operator, which is quite expensive, thus difficult in usage in daily clinical practice. The method is based on the attenuation characteristics of tissues exposed to X-rays at two peak energies (Lifshitz et al. 2016). The procedure remains safe for subjects of all ages, and ethnic groups in research and clinical settings. However, since there is some debate regarding whether low-level DXA radiation exposure to the patient provides a significant risk for their health, running multiple DXA measurements in children must be done with caution (Mattar et al. 2011b).

Different algorithms and equations are available to estimate the components of body weight, using several physical and biological models. Many studies have assessed body composition and nutritional status in patients with AN using or comparing DXA with other methods. It is vital to mention that through the DXA method it is difficult to record small changes in body composition, such as during a medical treatment or an intervention nutrition program, in general. For example, if the change in %BF is of the order of up to 3%, then it coincides with the limit of the long-term reliability and accuracy of the method (Lohman et al. 2000). Especially for obese or malnutrition individuals, the reliability and accuracy of the method is further reduced, therefore the measurement changes become problematic. Moreover, a small difference in calibration could be led to nonstandard results in AN patients (Mattar et al. 2011b).

One of the major difficulties in using this technology is related to the hydration of the individuals. It has been noted that a 5% change in FFM hydration (the usual hydration of FFM is ≈73%) changes the estimate of %BF by up to 2.5% (Lohman et al. 2000). This error rate is added to the error already inherent in the method. Thus, while a simple estimate of a patient's %BF, for example, is acceptable with the DXA method (knowing that the method has an error anyway), this method is not recommended for the assessment of possible changes in the body composition of patients with variable hydration.

DXA has been compared with other methods in several studies with AN patients. A recent study compared the body composition of 80 female AN patient (they were recruited from inpatient eating disorder units) with DXA and skinfold anthropometry to estimate %BF (Haas et al. 2013). Results indicated that, with an accepted reference 4C model in AN patients, skinfold measurements and DXA were similar in reliability. However, DXA is overestimated percentage of fat mass in healthy subjects. The authors refer that the comparison between an anthropometry, which is an anatomical model that

predicts body fat, with chemical models as DXA and the 4C model may be a limitation. Other studies, assessing body fat before and after weight restoration, report that ST measurements do not appear to be an alternative to DXA in estimating body fat percentage in adult patients (El Ghoch et al. 2012a, b). In the same way, in female adolescent, skinfold thicknesses (ST) equations do not accurately predict %BF when compared with the reference method of DXA (Haas et al. 2009). Therefore, these techniques could not be used interchangeably in this population (El Ghoch et al. 2014a**).** Haas et al. (2009) claim that DXA assessment may underestimate lean mass in underweight patients with AN, because patients who have a modest reduction in lean mass can be severely protein depleted (severe protein depletion measured by IVNAA seen in 17/50 AN patients). El Ghoch et al. (2014b) report that this effect is because starvation-induced changes in body composition may be associated with an increase in total body water, so direct methods may be more reliable means of assessing lean mass in underweight patients. However, Helba and Binkovitz (2009), in a review, evaluate the strengths and limitations of DXA as a pediatric BC method and consider the utilization of DXA to identify trends and variations in FM and LTM measurements in obese and anorexic children. They conclude that DXA is the most accurate and reliable method of determining body composition in children with AN and provides important information regarding %BF, regional FM, and LTM. Wells et al. (2015), but in a small sample size of ED patients, suggest that a multicomponent model may be more accurate for such longitudinal measurements than DXA.

Many studies evaluate the reliability of predictive BIA equations for FFM using DXA as reference method. Marra et al. (2018) show that the selected three predictive BIA equations considered exhibit an insufficient accuracy at the population and the individual level in 82 clinically stable restrictive AN patients attending the outpatient clinic. Also, all the BIA equations underestimated DXA-derived FFM. Few years earlier, Mattar et al. (2011b) compared measurements (50 female anorexic patients were included in the study) of FM and FFM done by DXA or by BIA using five different equations to identify the most suitable available BIA equation for AN patients. The best available BIA equation to calculate FFM and FM in patients with AN is found to be the Deurenberg equation which takes into account the weight, height, age of the patient, and BMI (12.8–21.0) as to age (13.4 and up to 36.9) (Deurenberg 2001). Although DXA is not as accurate as the 4C model, it is a widely accepted method for the measurement of body composition in clinical practice in AN patients.

Computed Tomography (CT)

Computed tomography imaging, also known as computerized axial tomography (CAT, CAT scanning) is a diagnostic imaging test used to create detailed images of organs, bones, soft tissue, and blood vessels. It is based on the absorption of X-rays. Although the CT is used mostly for diagnostic procedures, it is expensive and difficult in daily clinical practice; several studies have applied the technique for measuring abdominal and thigh fat, and thigh muscle mass in anorexic patients. Most of these studies have

used CT as a standard of reference. Bredella et al. (2010) refer that DXA underestimated abdominal fat in all groups and overestimated muscle mass in all groups, with larger main differences in subjects with higher weight. The difference between CT and DXA measurements became larger with increasing weight in premenopausal women, ranging from AN to obesity, but independently of the level of hydration. DXA can be used to assess body composition in AN patients. In AN, some studies used this method to evaluate fat mas and BC (subcutaneous and visceral adipose tissue). In 1989, a cross-sectional study by Mayo-Smith et al. (1989) shows that AN females aged 15–33 years tend to lose more subcutaneous fat than intra-abdominal fat compared to controls and the visceral adipose tissue to total adipose tissue was significantly higher in AN patients. Later, Gill et al. (2016) investigated the abdominal subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), thigh SAT, and thigh intermuscular adipose tissue cross-sectional area (CSA) in 40 premenopausal females with AN and healthy women with normal weights. The results demonstrated that females with AN have higher fat attenuation and lower fat cross-sectional area (CSA) than control group. Also, VAT attenuation, but not CSA, is inversely associated with lowest lifetime BMI, suggesting that fat attenuation assessed by using CT may serve as a biomarker of current and prior disease status in AN female (Gill et al. 2016). The radiation exposure of CT scans is high, and thus this method is used mainly for research purposes in AN patients and controls.

Ultrasound Measurement

The ultrasound technique has been used to assess fat thickness years ago and is one of the most promising methods for measuring fat layers in the body, particularly for the determination of subcutaneous adipose tissue. The ultrasound method allows fat thickness layer measurements with high accuracy to detailed information on fat patterning in underweight, normal weight, obese, clinical population, men, women, and athletes. The ultrasound technique does not involve radiation and may be advantageous against other imaging devices to quantify skinfold thickness. It has been studied as a low-cost, portable tool in nutrition assessment with a strong correlation with DEXA (Gomez-Perez et al. 2021).

Lackner et al. (2019) claim that the use of ultrasound measurement in AN patients with very low BMI, and similar BMIs, biochemical parameter indicators and carotenoid levels, showed enormous differences in SAT. Also, a new research of Lackner et al. (2021) demonstrates that high plasma β-carotene level is an indicator of delay in weight restoration in AN patients, and it is associated with reduced SAT levels.

More research, which includes reference methods like dual-energy X-ray absorptiometry, nuclear magnetic resonance, or BIA, is needed in clinical populations with AN patients. Methods as total body protein assessed with neutron activation analysis (Haas et al. 2018), total body water assessed with isotope dilution (Haas et al. 2013), hydrostatic weighing (Probst et al. 1996), and magnetic resonance imaging and spectroscopy (Vajapeyam et al. 2018) have been used in several researches to evaluate health parameters in ED population (Table 1).

Table 1 Methods to estimate body composition

Skinfold anthropometry	MM, FFM, fat distribution	Easy, low cost, portable, noninvasive, less affected by the hydration and water level	Low accuracy and sensitivity when nutritional interventions and practices are short Appropriate equation for clinical population
Dual energy X-ray absorptiometry	FM, LM, BMC, BMD, regional body fat	Reliable and valid tool for measuring body composition that uses the three-compartment chemical model Gives information for total and regional body fat and bone status	Hydration level of patients influence the results Low radiation exposure Not portable Cost Required experts Difficult to record small changes in body composition
BIA	TBW, FM, FFM, phase angle	Simplified technique that requires less operator training, portable, fast, inexpensive BIA parameters are useful in the detection of the state of starvation Phase angle can be used to evaluate the extracellular/intracellular water distribution in clinical population and is an accurate indicator of qualitative changes in body composition	Because hydration status and TBW are not constant, it is more accurate for patients after successive body weight restoration Possible sources of error are dehydration and body temperature
Computed tomography/ Magnetic response tomography	FM, fat distribution, MM	Create detailed images of organs, bones, soft tissue High accuracy	Radiation exposure is high High cost, not portable Required experts
Ultrasound measurement	**FM, MM,**	No radiation exposure and may be advantageous versus other imaging devices to quantify skinfold thickness Portable tool in nutrition assessment with a strong correlation with DEXA Reliable, valid, and fast method for assessing both subcutaneous and visceral adipose compartments	Requires experienced technician High cost More data for clinical population

Basal Metabolic Rate (BMR) and Resting Energy Expenditure (REE)

There is an interest in the research field in producing biomarkers that can accurately measure personal requirements as optimal nutritional therapy leading to better clinical outcomes (Achamrah et al. 2018b). There are three accepted methods of measuring energy needs including indirect and direct calorimetry, and non-calorimetry methods (i.e., equations) (Achamrah et al. 2018a, b; Levine 2005). Notably, assessment of both REE and BMR plays an important role in the dietary management. Although BMR and REE are used interchangeably, resting energy expenditure (REE) is defined less rigorously than BMR since the latter does not take into account variables like temperature or stress (Cuerda et al. 2007).

Basal metabolic rate (BMR) is the amount of energy expended of an individual while at rest in a neutrally temperate environment, in a postabsorptive state (which requires about 12 hours of fasting) and without physical or psychological stress. An accurate BMR measurement requires that a person's sympathetic nervous system is inactive, meaning that the person must be completely rested. But unfortunately, it is not regulated for body temperature and the level of stress. Indirect calorimetry methods, which are based on oxygen consumption and carbon dioxide production, are the gold standards for REE after an overnight fasting (Achamrah et al. 2018a, b). There are four main approaches for measuring REE through the use of indirect calorimetry, which are: a) total collection systems, like Douglas bag (Yoshida et al. 1981), b) open-circuit methods, c) confinement system, like respiratory chambers (Aulick et al. 1983), and d) closed-circuit systems (Mtaweh et al. 2018). Therefore, many hospitals are not equipped with the appropriate device for measuring direct calorimetry (Cuerda et al. 2007). On the other hand, there are three types of direct calorimetry, such as: a) isothermal systems, b) heat sink or adiabatic systems, and c) convection systems. Same to indirect calorimetry, direct calorimeter methods required also highly specialized laboratories and extremely expensive instruments (Levine 2005).

Total energy expenditure (TEE) is defined as the total amount of energy expended per day, and therefore it is determined by activity level and resting metabolic rate. Alternatively to calorimetry methods, doubly labeled water method is used for measuring TEE under free-living condition (Berman et al. 2015). This method provides an objective tool for assessing TEE, while not restricting subjects' activities (Livingstone and Black 2003).

Since indirect calorimetry methods are not easily applicable, it is recommended to use equations and disease-specific equations combining simple variables such as sex, age, height, and body weight (Ireton-Jones and Jones 1998). The most widely applied equations are Harris and Benedict and WHO/FAO/UNU. Harris and Benedict's equations predict BMR for men and women separately, combining variables like age, weight, and height in contrast to WHO/FAO/UNU where BMR is determined by age and includes only weight variable in the equation. Several equations

were also used, such as Owen, Mifflin, and Scalfi. The equation of Schebendach is used to correct Harris and Benedict equation for anorectics, and it was also suggested after determining that the intended BMR was lower than the measured BMR in patients with acute AN (Forman-Hoffman et al. 2006).

Many surveys demonstrate that individuals with anorexia nervosa have low or very low BMRs, due to their weight reduction and their loss of metabolically active tissue (~65%), as well as to their decrease in the metabolic rate of the remaining active tissue (Smith 2021). The basal metabolic rate on admission is invariably low, but it begins to increase shortly after the beginning of the nutritional rehabilitation process (de Zwaan et al. 2002). Several studies compared the accuracy of direct or indirect methods to estimated methods (using equations) in determining the BMR and REE (Table 2)

Predictive Equations

In general, as we know, many of the common equations do not predict truthfully BMR in the severe phase of AN. Also, it is notable that, the equations used in most studies were applied in anorexic patients with different anthropometric characteristics, in a fairly large range of age and with different severity of the disorder. It is also important to mention that, in many studies, the connection between body weight and BMR is notoriously modified. Additionally, Forman-Hoffman et al. (2006) claim that refeeding period demands to have under consideration both baseline needs and metabolic changes that follow nutrition recovery. The fact is having an accurate equation to estimate BMR in severe phase and during refeeding period will permit to specialist to evaluate patient's energy needs and patients to get weight slow and progressive.

However, researchers suggest that the Harris-Benedict, WHO/FAO/UNU, and Schofield equations overestimate BMR in underweight patients. They refer that using the Harris-Benedict equation to evaluate the energy need for refeeding underweight AN patients may result to suggest a hyperbolic energy intake. It appears to be appropriate only when the weight normalizes, and the situation has stabilized.

The Schebendach correction does not look to be fully adequate, mainly for adults patients (>18 years and BMI= 15,6±1,9), which widely underestimated BMP (Scalfi et al. 2002), whereas Marra et al. (2002) and Cuerda et al. (2007) claim that the predicted BRM of Schebendach formula was in close agreement with actual BMR in a wide range of age and predicts BMR in female adolescents (15,4±1,9), but not in young adult women.

FitMate method and the Müller equation seem to estimate REE in severe AN patients after short-term refeeding (> 18 years) satisfactorily. The equation proposed by Scalfi et al (2001) seem to be specific to estimate BMR in chronically underfed adolescents and young women patients.

Owen and Miffin-St. Joer equations appear quite accurate (within 10%) to estimate BMR at the baseline and few weeks of re-nutrition (Table 3).

Table 2 Studies in patients with anorexia nervosa using different equation measuring BMR reporting outcomes

Author (Year)	Sample	Measurement of BMR	Intervention	Estimation of BMR	Results
Scalfi et al. (2002)	n=120 AN (according to DSM-IV criteria) Two groups: a) n=34 adolescent girls aged 13 ± 17 years old; BMI = 15.4±1.9 kg=m^2), and b) n=86 young adult women 18–30 years old (BMI = 15.0±1.9 kg=m^2)	BMR was measured by indirect calorimetry. Energy expenditure was then calculated with the abbreviated Weir's formula	Does not include nutritional intervention	BMR predicted by the Schebendach formula	In the adolescent group Schebendach predicted BMR was 3,589±448 kJ=day and mean predicted measured difference was only 9kJ=day In the young-adult group the Schebendach equation significantly underestimated measured BMR by 362 kJ=day with a wide range between the 95% limits of agreement (2270 kJ=day)
Marra et al. (2002)	n=237 AN (according to DSM-IV criteria) Adolescent group n=43, 13–17 years old Young-adult group n=194, 18–40 years old	BMR determined by open-circuit indirect calorimetry according to standardized conditions (using either a canopy system in Naples or Douglas bags in Rome) BMR of adolescent group: 3,658±665 kJ/day BMR of young-adult group: 3,907±760 kJ/day	Does not include nutritional intervention	Equations were used: a) WHO/FAO/UNU, b) Harris and Benedict, or c) the formula proposed by Schebendach et al. to correct the Harris-Benedict estimates for anorectics	WHO/FAO/UNU and Harris-Benedict equations overestimated BMR by +27% and +39%, respectively, in the sample as a whole, whereas the predicted BRM of Schebendach formula was in close agreement with actual BMR (1±12%) Adolescent group: higher predicted values of

(continued)

Table 2 (continued)

Author (Year)	Sample	Measurement of BMR	Intervention	Estimation of BMR	Results
					WHO/FAO/ UNU (5124±254 kJ/day) and Harris-Benedict (5242±241 kJ/day) than the actual values ($p<0.001$), very similar (3638±395 kJ/day) predicted values and not statistically different with the Schebendach formula Young-adult group: higher predicted values of WHO/FAO/ UNU (4,602±384 kJ/day) and Harris-Benedict (5,158±292 kJ/day) than the actual values ($p<0.001$), whereas the Schebendach formula was on average underestimated BMR (3477±537kJ/day, $p<0.001$)
Forman-Hoffman et al. (2006)	n=7 AN (according to DSM-IV criteria) 19–40 years old; mean BMI (at baseline) was 14.4 ± 1.3 kg/m2	REE was measured by indirect calorimeter (Datex-Ohmeda; Deltatrac) using a computerized flow-through, canopy-gas	Treatment goal of refeeding period was 3,500 kcal/day (all participants reached this goal)	Predicted BMR was calculated using ten different equations: Harris-Benedict, Schebendach, and Mifflin estimated BMR	At baseline: The most accurate equation was the Mifflin which overestimated BMR by a mean of 5.7%; the Owen equation overestimated

	BMI was calculated at each time point	analyzer system At baseline: Median BMR (1,000 kcal/day) was significantly lower than median BMR at 3 weeks post-refeeding (1,220 kcal/day) The median change in BMR was 182 kcal/day ($p = 0.02$)		incorporated weight, height, and age into their equations; six equations of Scalfi, three of them incorporate weight and age into their BMR estimating equations, while the other three incorporate only weight into their BMR estimating equations as Owen equation too	BMR by a mean of 8.9%; Harris-Benedict equation overestimated BMR by a mean of 21.5%; the Schebendach and Scalfi formulas underestimated BMR by a mean ranging from 14.6–25.3% At 3-week follow-up: The most accurate equation was the Harris-Benedict equation which overestimated BMR by a median of 1.8%. The Scalfi and Schebendach formulas greatly underestimated post-refeeding BMR by a median range of 17.9% to 32.0%, whereas Owen equation underestimated BMR by a median of 8.7%
Konrad et al. (2007)	n=10 (8 of them met the diagnostic criteria for anorexia nervosa restricting type and 2 were diagnosed with binging/purging type) Mean age was 31±12.87 y.o., 9 were Caucasian	RMR was measured weekly by calorimeter that measures oxygen consumption (VO2) using the Weir equation and a constant respiratory quotient (i.e., VCO2/VO2)	Does not include nutritional intervention	RMR predicted by Harris-Benedict equation	H-S equation overestimated RMR on admission compared to measured RMR (1200.22±72.73 and 1005.0±131.34, respectively)

(continued)

Table 2 (continued)

Author (Year)	Sample	Measurement of BMR	Intervention	Estimation of BMR	Results
	females and 1 was Asian American				
El Ghoch et al. (2012a, b)	N= 39 AN (according to DSM-IV criteria) 13–45 years old Mean of BMI was 14.39 (1.75) (64.1% of the participants having a BMI=15.0)	REE was assessed by SWA (by the Douglas bag method)	Data were collected on the third day of admission after 24 h of refeeding, with a diet of 1.500 kcal (carbohydrate 50%, protein 20%, and fat 30%) composed of conventional foods and divided into four standard meals	REE estimated by: a) indirect calorimetry using the FitMate method and b) Muller equation (for individuals with a BMI<18.5)	No significant differences between the mean REE values estimated with the Douglas bag method and the mean values estimated with the FitMate method The mean REE estimated with FitMate method, Muller et al.'s equation and SWA were 962.56 kcal/day (200.74), 916.76 kcal/day (111.35), and 1073.77 kcal/day (132.27), respectively SWA is not interchangeable with either the FitMatee method or the Muller equation in assessing REE in patients with AN

Kochavi et al. (2020)	n=60 (n=35 diagnosed with AN, n=29 diagnosed with AN-R and 6 with AN-B/P, n=25 with BN, according to the DSM-5 criteria)	REE was measured with the Deltatrac indirect calorimeter (assessed 2–4 weeks from admission and at discharge, when the patients achieved stabilization of weight and disordered eating)	To maintain weight stabilization, AN patients consumed an average of 2,000–2,500 kcal/day (50% carbohydrates, 30% of fat, and 20% of protein)	Expected REE calculated by Harris-Benedict equation. Expected REE was significantly greater than measured REE between these two time points	AN patients: (at admission) expected REE was 1,276.40 ± 93.8 kcal whereas measured REE was 972.60 ± 109.2 (p = .001); (at discharge) expected REE was 1,417.40 ± 102.8 kcal whereas measured REE was 1,255.40 ±162.8 (p = .001) BN patients: (at admission) expected REE was 1,390.50 ± 126.7 kcal, whereas measured REE was 1,102.80 ± 162.8 (p = .001); (at discharge) expected REE was 1,438.20 ± 80.3 kcal, whereas measured REE was 1,207.60 ± 136.6 (p = .001)

Table 3 Equations for the prediction of BMR in the AN patients

Equation	Formula	
	Men	Women
Harris-Benedict (Kcal/day)	BMR: 66.47 + [13.75 × weight (kg)] + [5 × height (cm)] – [6.75 × age (years)] (13)	BMR: 665.1 + [9.56 × weight (kg)] + [1.85 × height (cm)] – [4.68 × age (years)]
Owen (Kcal/day)	BMR: 879 + [10.2 × weight (kg)]	BMR: 795 + [7.18 × weight (kg)]
Schebendach (kcal / day)		BMR: (-1435 × 4.186) +1.84 x Harris-Benedict
Miffin-St. Joer(kcal / day)	BMR = 10 * weight (kg) + 6.25 * height (cm) – 5 * age (y) + 5 (kcal / day)	BMR = 10 * weight (kg) + 6.25 * height (cm) – 5 * age (y) -161 (kcal / day)
Schofield (kcal/day)	10–18 17.686 × W (kg)+ 658.2 18–30 15.057 × W (Kg)+ 692.2 30–60 11.472 × W (kg)+ 873.1	10–18 13.384 × W(kg) + 692.6 18–30 14.818 × W(kg) + 486.6 30–60 8.126 × W(kg) + 845.6
Scalfi (Kj/day)		Adolescents' patients BMR=92,8Xweight Young Adolescents BMR=96,3Xweight

Anthropometric Measurements

Body Weight and Body Mass Index

The measurement of body weight is not in itself a reliable tool for determining the nutritional requirements in AN, regardless of age and nutritional status (Mattar et al. 2011a, b). Nonetheless, it is an eessential and an easy tool to use in the starting and in the post-nutritional intervention evaluation. Changes in body weight may represent alterations in body cell mass, fluid balance, or a combination of these and so, from a nutritional perspective, they provide limited information (Probst and Goris 2012; Madden and Smith 2016).

Body mass index (BMI) is a simple anthropometry index and is widely used in public health and clinical nutrition to provide a quick overview of nutritional well-being and status. It is assessing obesity and malnutrition (World Health Organization 2014). The WHO classification of BMI describes 11 principal categories ranging from severe thinness to obesity class III. Normal range defined as 18.50–24.99 kg/m2 (calculation of BMI requires reliable measures of body weight and standing height).

BMI illustrates actual changes in ratio of weight to height and is highly helpful in monitoring the treatment of obesity. In clinical population, such as anorexia nervosa, changes in BMI may not be linked to clinical improvement of health-related

outcomes, in comparison to different body composition measures and age (Nagata et al. 2017; Engelhardt et al. 2021). In addition, because BMI considers the body as a whole rather than regionally, BMI is a poor index of fatness in females with ED, explaining only ~50% of the variance in percentage fat estimated by skinfolds or densitometry. Also, it is unable to identify where body fat is located. Fat mass and fat-free mass are clinically more essential for assessing the severity of the disorder and its response to nutritional treatment (Probst et al. 1996; El Ghoch et al. 2017; Speranza et al. 2021).

Fat-Free Mass Index

The fat-free mass index (FFMI) is an alternative to BMI, which evaluates a person's muscle mass. Several researchers support that FFMI may be a useful tool in a nutritional assessment to evaluate lean tissue. It is an index which helps assessing body composition when BMI is not a sufficient indicator in individuals who have a similar body composition, but differ in height allowing identification of those suffering from malnutrition, or those that possess a relatively high muscle mass (Shypailo and Wong 2020). Fat mass index is calculated by dividing fat weight in kilograms by height in meters squared. The formula is FFM index: FFM (kg)/length $(m)^2$. Values <15 kg/m2 in women and <17 kg/m2 in men, based on data from healthy adults, are proposed as cutoffs for diagnosing undernutrition when accompanied by unintentional weight loss (Nicholls et al. 2002).

Waist-to-Hip Ratio and Waist Circumference

The waist-to-hip ratio (WHtR) and waist circumference (WC) are quick and easy measures of fat distribution and are used as an indicator of central fat distribution that may help illustrate a person's overall health, fertility, and the risk of developing serious health conditions (WHO 2011; Madden and Smith 2016). They have been shown, also, to be better predictors of mortality than BMI at population level. The WHO informed that a healthy WHtR is <0.85 or less for women and 0.9 or less for men. High risk is defined by a WC> 102 cm for men and > 88 cm for women. There is limited information and studies about the usefulness of the WHtR and WC in anorexic patients, especially during recovery or after weight gain, and on the relationship between waist-to-height ratio and the procedure of recovery.

Studies indicate the existence of adipose distribution abnormalities and increased relative central adiposity on weight restoration in adult AN patients. It has been demonstrated, also, that adult females lose more peripheral (subcutaneous, extremity), rather than central, body fat (visceral, trunk, android) during their illness and female adolescence lose more central (trunk, visceral) than peripheral body fat (subcutaneous, extremity), but the data is not so strong for males. More studies are needed to investigate and specify body fat distribution before and after long-term complete weight restoration in adolescent and adult males (Orphanidou et al. 1997;

Scalfi et al. 2002; Mayer et al. 2005). Furthermore, findings display that in adolescent women with AN, weight recovery results in a tendency toward normalization of adipose tissue mass and not of increased central adiposity (Misra et al. 2003). Additionally, data suggest that adolescent females with AN tend to lose more central/visceral fat (trunk) than peripheral fat (subcutaneous), while adolescent males seem to lose more peripheral fat (El Ghoch et al. 2014a; Hübel et al. 2019). Mayer et al. (2005) refer normalization of weight in the short term in adult women with AN, this abnormal distribution appears to normalize within a one-year period of weight maintenance. There is less information about after long-term maintenance of complete weight restoration. Results show that after short-term weight restoration, whether partial or complete, adult females with AN tend to accumulate trunk fat, which contributes to a predominantly central distribution of body fat (El Ghoch et al. 2014b).

Changes in circumferences in thigh and mid-upper arm correlate significantly with an increase in weight, BMI, FM, and FFM in anorexic female adolescences (Martin et al. 2009; Konstantynowicz et al. 2011). Circumferences could be useful, also, for assessing muscle status in patients during the treatment process (Konstantynowicz et al. 2011; Fernandez-del-Valle et al. 2015). Weber et al. (2020) refer that a thigh circumference >50 cm was significantly connected to BMI>18 and a beneficial outcome in a sample of 258 females and 11 males with anorexia, and the measurement of body circumferences in addition to weight is a reliable tool when monitoring the treatment of AN.

Thigh and mid-upper arm circumference might work as a useful predictor of body fatness in AN, particularly if the availability of equipment measuring body composition is limited and should therefore be further evaluated in independent studies. Hip circumference also provides an indication of adiposity, although its value in predicting health risk is unclear for all-cause mortality. So, WHtR and WC are additional anthropometric measures in AN, and would be better to be combined with other measurements.

Skinfold Anthropometry

Skinfold anthropometry is an indirect, simple method to assess body composition and is widely used in healthy and clinical populations. The body is divided into two parameters, the body fat mass and fat-free mass, based on different equations and/or number of anatomical sites. This technique is more used in lean and normal weight persons. Skinfold measures are taken with an inexpensive instrument called "caliper." Several calipers are disposed, which may differ in accuracy. Many combinations of skinfold measures are proposed by the experts to estimate body fat. Triceps, subscapular, suprailiac, and abdomen are the most common. Triceps skinfold is most often used in nutritional assessment. All skinfold measurements should be taken by trained personnel on the right side of the body, with the subject standing upright, and should be evaluated against reference values. The method is noninvasive and does not require the use of electronic devices (Madden and Smith 2016).

It is important to emphasize that in addition to the inability to assess abdominal fat, skinfold thickness measurements mistakenly assume that subcutaneous fat, measured at one or more selected sites, measures total body fat stores (Eaton-Evans 2013). Skinfold anthropometry has a low accuracy and sensitivity when nutritional interventions and practices are short. This method has been used in anorexic populations for more than five decades (Stonehill and Nunnerley 1971). The fact that it is both less affected by the hydration and water level and is easy to be used under any circumstances makes this method advantageous for use in AN patients.

Many studies compared skinfold thicknesses with other methods. As a method, skinfold thickness equation appears to be as accurate as underwater weighing, in a large sample of female AN patients, adolescents, and adults, before and after weight gain (Probst et al. 2001).

Popiołek et al. (2019) findings suggest a correlation between BIA parameters and anthropometrical measurements, such as circumference and skinfold measurements, in 46 AN female patients at the beginning of their hospitalization.

Also, changes in skinfolds at biceps, triceps, and abdominal sites are correlated well with the increase in weight, BMI, or FM in female AN patients (Scalfi et al. 2002) and in response to resistance training when performed after hospitalization (Fernandez-del-Valle et al. 2015). But it is not recommended for anorexic patients with a BMI <15 kg/m^2 as it overestimates fat mass and underestimates body fat when compared with DXA in adults with an eating disorder, both before and after weight gain (El Ghoch et al. 2012a, b). The accuracy and validity of the adductor pollicis muscle thickness in patients with AN require further studies (Soto-Célix et al. 2019). Many biomarkers have been investigated for their part in diagnostics and prognosis of AN, making an effort to comprehend which particular factors influence the abnormal behaviors and attitudes toward food among AN patients.

Surrogate Measures

Self-Reported Measurements

Fact remains that it is difficult to learn about eating habits especially in eating disorder patients, but the accurate specifying of energy, as well as macro- and micronutrient intake is crucial for nutritional evaluation and the development of dietary practices, guidelines, and recommendations in AN, as AN patients appear to become hypermetabolic during weight restoration. Also, this information can contribute and assist patients to normalize their dietary habits (Marzola et al. 2013). Nutritionists and clinical dietitians count widely on self-reported measures of dietary intake to estimate food and nutrient consumption at individual level (Schebendach et al. 2012a, b; McMaster et al. 2021). Although, there are numerous disadvantages of self-report measures that threaten the reliability and validity of measurement, the major advantage is that it is a simple way to collect information and elements at a low cost, fast, and in situations and locations the researchers wish. Combined with

additional data, self-reported measurements provide, also, the appropriate background to assign realistic and objective nutritional education goals (Assessment FD 2018). Direct assessment of the dietary intake of anorexics, especially in the phase of recovery, may be very informative in the evaluation of the intervention at a qualitative level and a core component of the developed intervention (Gibson 2005; McMaster et al. 2021). Self-reported measurements, suggested by researchers, come from questionnaires and food records (Beaumont et al. 1981; Hadigan et al. 2000; Schebendach et al. 2012a, b; Baskaran et al. 2017).

Retrospective Methods Measure Food Intake

A 24-Hour Dietary Recall (DR)

A 24-hour dietary recall is an interview, using an open-ended format, intended to collect detailed information about quantity and quality of foods and beverages respondents have eaten the last 24 hours in their home. The interview is taken by an expert nutritionist who has been trained in interviewing techniques. The use of a four- or seven-day food recall (including one weekend day) is beneficial as a tool for selecting the appropriate food items to be included in the food frequency questionnaire and helpful as an instrument for obtaining additional information.

Food Frequency Questionnaire

The food frequency questionnaire (FFQ) is used to assess eating habits of the respondents. Although this method may be less accurate than weighed dietary records and 24-hour or 4-day dietary recall or 24-hour record, it is a simple and validated tool to assess the habitual dietary patterns of the responders (Gibson 2005; Taylor et al. 2009; Willett 2012). Specifically, the FFQ assesses the frequency with which foods, beverages, sweets, or any other categories of foods of interest to the nutritionist, eaten over a certain time (daily, weekly, or monthly), and may reveal nutrition habits not evident from a food record. It offers mostly qualitative information about the intake of the responder who complete the questionnaire (Taylor et al. 2009).

The lists of food suggested in the FFQ are close ended. Depending on the purpose of the research, they can include information about portion sizes and/or quantity of food intake, details about cooking, and preparation methods. Thus, FFQ is regarded as a quantitative questionnaire because it depicts the amount of food ingested.

It is substantial to notice that FFQ requires good memory from the respondent and, therefore, can give incorrect outcomes for people with impaired cognitive functions or declined cognitive/mental ability. In adolescent period parents tend to complete the questionnaire on behalf of their children. A combination of self-reports measurements, such as FFQ with DRs or 24HR, with other biomarkers, have recently been proposed by researchers to obtain more accurate estimates of dietary intakes in a clinical population and in individual level.

Prospective Methods Measure Food Intake

A 24-Hour Record

A 24-hour or more days record is a preferred method, when diet counseling or correlation of intakes with biological parameters is involved (Chiurazzi et al. 2017). Through this method one collects information by responders' self-record at the time the food, beverages, and fluids are eaten in real time (Taylor et al. 2009). Also, a 24-hour or more days record informs the experts about the food frequency consumption, meals' structures, diet variety, and quality of dietary choice (Schebendach et al. 2012a). By means of this method, the collector of information may eliminate reliance on a responders' memory. To obtain accurate data, however, respondents must be trained before participating in the evaluation. The 24-hours dietary recall is usually fulfilled by mothers or fathers, in the case of children.

Both methods, 24-hour dietary recall and 24-hour record, are concentrated on short-term intake, but short-term dietary report is especially of interest when investigating patients with AN. Especially, at the level of energy and protein intake.

One of the most difficult points of these methods of recording food is the estimation of the exact amount consumed by the responder (Schebendach et al. 2011). In AN, the dietary record shows a higher energy intake than real, particularly when patients tend to overreport intake (Hadigan et al. 2000). A number of methods exist to estimate the ingested amounts. One of them requires double weight; the responders weigh food to eat and then weigh uneaten. However, this method is not recommended for patients with ED, who are already quite concerned about the amount and volume of food they consume. The use of a spoon, a plate, the nutritional label of the product, a ladle, and images and digital photographs may also be suggested to facilitate the patient to record the amount of food intake (Rosenvinge and Pettersen 2012).

Several studies compare dietary assessment by traditional methods versus innovative technologies. Some of them argue that electronic records would be a useful tool both for epidemiological studies and in the clinical environment. Also, mobile phone applications may be used to replace the 24-h recall and serve as feasible tools for dieticians investigating dietary intake at a population level (Vasiloglou et al. 2020).

The new technologies are a reality and have advantages, but to our knowledge there are no studies available suggesting their use in AN. There is a need for well-designed long-term studies to explore and analyze the combination of traditional methods and state-of-the-art technological tools which characterize the new era of nutritional assessment (Fig. 2, Table 4).

Other Biomarkers

Several biomarkers have been investigated for their contribution to diagnosis, prognosis, treatment, and management of AN, independently of weight status, including bone mineral density, gut microbiome, and hormones. The study of

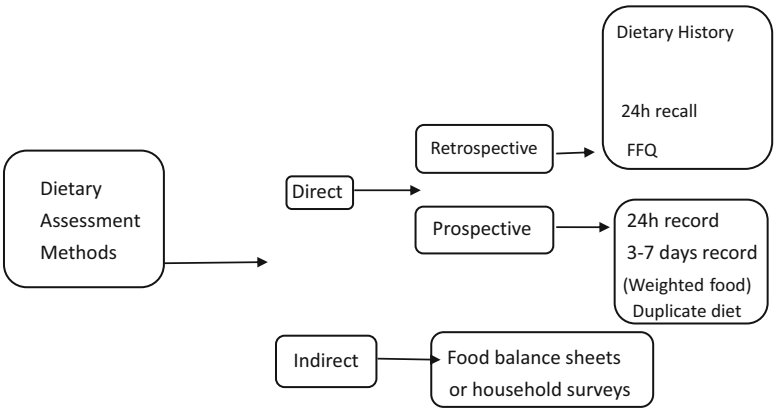

Fig. 2 Dietary assessment methods to estimate food and nutrient consumption at general and individual level

these biomarkers in AN patient may enable researches to comprehend better their abnormal behaviors and attitudes toward food, as well as to evaluate more accurate the effects of weight restoration intervention programs on them. With further development, clinical tests for these biomarkers may have utility in screening individuals who may be underweight due to AN or monitoring the effects of weight restoration interventions (Caso et al. 2020; Grzelak et al. 2018; Kim et al. 2021; Ruusunen et al. 2019; Seidel et al. 2021) (Table 5).

Mini-Dictionary of Terms

- Anorexia nervosa is a severe chronic mental disorder with a high mortality rate caused by severe malnutrition. It is characterized by dangerously low body weight and various other health problems.
- Anthropometric measurements outline body mass, body mass index, weight, body fat, and fat-free mass index.
- Basal metabolic rate *is* the amount of energy expended of an individual while at rest in a neutrally temperate environment without physical activity.
- Body composition expresses the amount of fat mass, muscle mass, bone, and water level in the body.
- Indirect methods of body composition: anthropometry, skinfolds, circumference, and BIA.
- Nutritional biomarker refers to any objective measure that give information about health status of a person involving nutrition attitude and behavior. It Illustrates biological consequence of a diet.
- Nutritional status is the condition of the body in response to and influence by intake and utilization of nutrients and diet. It is an indicator of health status in individual or general level.

Table 4 Dietary assessment methods

	24-h dietary recall	24-h dietary record / Dietary record	Food frequency questionnaire	Innovativemtechnologies
Methods	It is an interview, using an open-ended format by an expert Subjective measure	It uses an open-ended self-report questionnaire Subjective measure	Questionnaire that includes a food list, usually close ended. It can be self-, or interviewer administered It is a semiquantitative questionnaire	Digital mobile-based technologies personal digital assistants, mobile phones, interactive computer software scan- and sensor-based technologies
Collected information	Collects detailed information about quantity and quality of foods and beverages respondents eaten the last 24 hours	Collects information by responders' self-record at the time the food, beverages, and fluids are eaten in real time The number of days depends on the purpose of the collection	Collects information by responder's self-report or by an expert over a relative long period	Collects information by responder's self-report or by an expert
Limitations	Based on respondent's memory Requires well-trained interviewers and demand time spent on data entry and food matching with food composition data Based on respondent's ability to describe the food and to evaluate	Based on respondent's motivation Demand time spent on data entry and food matching with food composition data Respondents may ignore to record certain food Accuracy of records decreases over time (in case of	Based on respondent's motivation The food list may be not meet all the foods eaten by the respondent Requires abilities by the respondents, e.g., memory, numeracy skills, if self-reported Does not provide accurate information on portion size eaten if it is not semiquantitative	Requires certain level of technological knowledge Requires Internet access (for real-time data collection) Scanning barcodes is applicable to packed food only

(continued)

Table 4 (continued)

	24-h dietary recall	24-h dietary record / Dietary record	Food frequency questionnaire	Innovativemtechnologies
	portion size Recall bias may be present	more than one day record) Less accurately recorded food intake in children		
Strengths	Assesses the usual and detailed intakes Open-ended format used is suitable for all dietary patterns There is less load on the respondent's memory resulting in accuracy	Useful in assessing detailed food and nutrient intakes at individual level Appropriate for all dietary patterns Does not based on respondent's memory Allows for true time portion size estimation	Evaluate usual dietary intake over a long period Does not influence dietary habits Interview-based FFQ does not based on the numeracy skills of the respondent Collects information about cooking and preparation methods The number of food items in the list is proportionate by persons or population characteristics and study's aim	Do not based on respondent's memory Easy to use Objective dietary evaluation

- Nutritional/nutrition counseling is a crucial procedure of patients care. The professional/dietitian evaluates the nutritional status of a patient and identifies fields where changes are needed. Then a dietitian gives the correct and appropriate instructions to help patients to apply and keep the required dietary adjustments.
- Resting energy expenditure is the amount of energy of individual while at rest but it does not consider the temperature, physical activity, and stress.
- Self-report questionnaires: It is one of the most widely used nutritional assessment strategies with several advantages. It allows the professional to collect

Table 5 Proposal surrogate biomarkers in anorexia nervosa

Biomarker	Claims
Support vector machine technique	Neuroimaging biomarkers, allows to accurately classify individuals with ED Differentiate different ED phenotypes Monitoring disease progression
Bone mineral density	Estimate of starvation status
Interleukin-6(*IL-6) (*pleiotropic proinflammatory cytokine) Cytokines are involved in processes in the metabolism and behavior	IL-6 concentration is higher in AN patients than healthy populations Extensive exercise may be is associated with the overall state of the immune system in AN patients Reduction may be associated with appetite regulation, illness severity, regulation, and function of adipose tissue, including both visceral and subcutaneous fat The IL-6 serum concentrations elevated in AN and "normalize" during the treatment Further studies on the neuroimmunological response in AN patients are needed
Tumor necrosis factor alpha (TNF-α, cytokine) (Patel and Patel, 2017)	Increased production of TNF-a lead to weight, skeletal muscle, and adipose tissue loss Correlate with body weigh/psychological parameters
Gut microbiome (microbial composition)	Reduction in microbial diversity is correlated with reduced immune system and decreased ability for energy harvest and fat storage from the foods Nutritional rehabilitation and weight gain is associated with elevated levels of specific bacteria It is still not obvious whether microbial variations are a resultant of chronic caloric restriction, or a relevant factor
Leptin (inform the brain about fat storage)	Serum leptin concentrations are low in the acute anorexic state and is correlated to BMI Changes in leptin secretion are associated with energy intake and REE and is associated with weight gain Low serum leptin concentration is associated with the adaptation to starvation as lowered T_3 and REE Low serum leptin concertation is correlated with energy intake Increased leptin secretion rate during weight restoration is related to changes in BMI and not to FM. Weight gain depends on the severity of starvation Low leptin concentrations but a normal leptin secretion rate in severely malnourished women with AN Low serum concertation, paired with BMI, is more effective than BMI alone in predicting AN diagnosis Leptin changes are differently associated with sex

(continued)

Table 5 (continued)

Biomarker	Claims
Ghrelin	It could be used as an index of treatment and recovery status. Elevated levels of ghrelin are associated with the acute state of AN
Neuropeptide B (NPB) vaspin (VAS) levels and total antioxidant status (TAS)	Higher-level (in comparison to control) NPB and VAS concentrations and lower values of TAS levels in AN patients Adjusted serum neuropeptide B level according to BM compared to parallel adjusted serum vaspin and TAS levels may be significant in predicting AN diagnosis Increased NPB levels are not affected by body weight normalization after hospitalization TAS and VAS in the saliva may be a reliable noninvasive source of data for strong nutritional biomarkers

accurate information about a participant's eating habits for the past and the present easily and without cost.

- Total body counting and neutron activation is a direct method that measures in vivo the amount of naturally radioactive elements (K, N, P, H, O, C, Na, Cl, and Ca) in the body. From the measured radioactive elements levels, fat-free mass can then be estimated.
- Total body water is a direct method to evaluate body composition at the molecular level in which an individual consumes a certain isotope dilution that can be measured in the body.

Summary Points

- There is evidence that AN is the ED which has the most serious consequences in a person's life with organic, behavioral, psychological, and social consequences.
- It is very important before any nutritional approaches to evaluate, as correctly as possible, caloric needs, energy expenditure, and nutritional status.
- The researchers are very interested in producing biomarkers that can accurately measure personal requirements as optimal nutritional therapy has been recently associated with better clinical outcomes.
- Many surveys demonstrate that individuals with anorexia nervosa have low or very low BMRs, due to the weight reduction and the loss of metabolically active tissue.
- A variety of equations have been investigated for their accuracy of estimating energy needs in AN patients.
- The most used equations do not predict truthfully BMR in the severe phase of AN.

- Assessment of nutritional status which relies on body composition and surrogate measures is vital for the evaluation of the general health status of AN patients.
- Many methods and techniques, such as anthropometry, BIA, DXA, computed tomography, as well as inflammatory and hormonal biomarkers have been investigated for their role in the early diagnosis and treatment of AN.
- Despite DXA is not as accurate as a 4C model, it is a widely accepted method for the measurement of body composition.
- BIA may be more accurate for patients with successive clinical renourishment. It's use in patients with BMI$<$15 kg/m^2 is not recommended, because they underestimate FM.

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Part VII

Resources

Recommended Resources for Biomarkers in Nutrition: Methods, Discoveries, and Applications

54

Rajkumar Rajendram, Daniel Gyamfi, Vinood B. Patel, and Victor R. Preedy

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Abstract

Nutritional status reflects supply and demand. Optimum nutritional requires sufficient nutrients to meet daily requirements. Dietary intake can be used to measure nutritional status but such records are subjective and strongly affected by recall bias. Thus, more accurate tools that can quantify nutritional status objectively are required. A biomarker of nutrition can be used to indicate nutritional status. Several different nutritional biomarkers are required to elucidate the complex relationship between health and nutrition. Several biomarkers are currently used in routine

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clinical practice. It can be argued that there is an ongoing dialogue as to the best biomarkers for specific nutrients or groups of nutrients in different scenarios or populations. Thus, the application of nutritional biomarkers is actively being investigated and the understanding of this topic has advanced in recent years. However, keeping abreast of current research can be problematic so we have compiled tables of the resources recommended by active practitioners and researchers. These include information on regulatory bodies, societies, organizations, and other resources.

Keywords

Books · Evidence · Journals · Development · Professional societies · Regulatory bodies

Introduction

Nutritional status reflects the balance between nutrient supply and demand (Picó et al. 2019). Optimal nutritional status requires the consumption of sufficient nutrients to meet the body's daily requirements (Picó et al. 2019). This enables a variety of cellular processes to occur, including development, growth (Picó et al. 2019), and adaptations to the aging process (Preedy 2014).

Measures of dietary intake can be used in nutritional studies (Thompson et al. 2010) and tools are available to screen for malnutrition (Rajendram and Khan 2019). However, food records or frequency questionnaires are very subjective tools that are strongly affected by recall biases (Frobisher and Maxwell 2003). This may be particularly challenging in those at greatest risk of malnutrition (e.g., alcohol misusers Rajendram and Preedy 2008). In any case, dietary intake per se does not impart information on nutritional status as this will be influenced by absorption (or malabsorption) and metabolic utilization. Thus, more accurate tools that can quantify nutritional status or dietary intakes are required (Picó et al. 2019).

Biomarkers can be defined as "characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention" (Atkinson et al. 2001). Biomarkers potentially have significant value in the assessment of nutritional status or dietary studies including the consumption of different foods.

Different biomarkers are required to elucidate the complex relationship between health and nutrition. These include markers of health/disease, intake, and function or effect (Corella and Ordovas 2015; Picó et al. 2019). However, there is no clear consensus on the best biomarkers for specific nutrients or specific disease processes, which are applicable in all scenarios.

The application of nutritional biomarkers is actively being investigated for a variety of dietary components and dietary-related processes (e.g., related to

linoleic acid (Li et al. 2020), chromium (Amini et al. 2021), aging (Leitao et al. 2022), inflammation (Koelman et al. 2022), cocoa products (Chen et al. 2022), whole grains (Hajihashemi et al. 2021), vitamin D (Cashman et al. 2022), and numerous other areas covered in Patel and Preedy (2022). Even experienced researchers and clinicians struggle to stay up-to-date. We have therefore produced tables containing resources as recommended by active researchers and practitioners, which draws upon the wealth of experience and acumen acquired over many years. The list below acknowledges all the experts who helped to prepare these valuable resources.

Resources

Tables 1, 2, 3, 4, and 5 list the most up-to-date information on the regulatory bodies (Table 1), professional societies (Table 2), books (Table 3), emerging technologies and platforms (Table 4), and other resources of interest (Table 5) that are relevant to an evidence-based approach to biomarkers of nutrition. Some organizations are listed in more than one table as they occasionally fulfill multiple roles.

Other Resources

The Wellcome Collection (https://wellcomecollection.org/collections) and The British Library (https://www.bl.uk/) also list material on topics related to biomarkers or nutrition.

Other chapters on resources relevant to biomarkers (recommended by authors and practitioners) may also be relevant to biomarkers of nutrition. These include nutrition and oxidative stress (Rajendram et al. 2020), maternal nutrition (Rajendram et al. 2017a), general aspects of biomarkers (Rajendram et al. 2016a), biomarkers of cardiovascular disease (Rajendram et al. 2016b), biomarkers of renal disease (Rajendram et al. 2017b), and aging (Rajendram et al. 2021).

Other chapters on resources relevant to nutrition (recommended by authors and practitioners) may also be relevant to biomarkers of nutrition. These include nutrition and the menopause (Rajendram et al. 2013), glutamine (Rajendram et al. 2014), branched chain amino acids (Rajendram et al. 2015), famine, starvation, and nutrient deprivation (Rajendram et al. 2018a), nutrition and epigenetics (Rajendram et al. 2018b), aging (Rajendram et al. 2021), diet and nutrition in critical care (Alzaid et al. 2015), and the metabolism and physiology of bariatric surgery (Rajendram et al. 2016c).

This list of materials in these tables is included to provide general information only. It does not constitute any recommendation or endorsement of the activities of these sites, facilities, or other resources listed in this chapter, by the authors or editors of this book.

Table 1 Regulatory bodies or organizations dealing with biomarkers or nutrition

Regulatory body or organization	Web address
Academy of Nutrition and Dietetics	https://www.eatright.org/
American College of Rheumatology	https://www.rheumatology.org
American Society of Parenteral and Enteral Nutrition	https://www.nutritioncare.org/
British Association for Parenteral and Enteral Nutrition	https://www.bapen.org.uk/
Centers for Disease Control and Prevention	https://www.cdc.gov/
Critical Care Nutrition	https://www.criticalcarenutrition.com/
Eurecat	https://eurecat.org/
European Commission's Food Safety	https://ec.europa.eu/food/overview_en/
European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN)	https://www.espghan.org/
European Society for Parenteral and Enteral Nutrition	https://www.espen.org/
Food and Drug Administration	https://www.fda.gov/
International Life Sciences Institute	https://ilsi.eu/
International Zinc Nutrition Consultative Group (IZINCG)	https://www.izincg.org/
National Health Service England	https://www.england.nhs.uk/
National Institute for Health and Care Excellence (NICE)	https://www.nice.org.uk/
National Institute on Aging	http://www.nia.nih.gov
Sociedad Española de Nutrición	https://www.sennutricion.org/es/inicio
Sociedad Española de Nutrición Clínica y Metabolismo	https://senpe.com/
Sociedad Mexicana de Nutrición y Endocrinología A.C.	https://endocrinologia.org.mx/
World Health Organization (WHO)	https://www.who.int/

This table lists the regulatory bodies and organizations involved with biomarkers or nutrition. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers of nutrition within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 2

Summary Points

Biomarkers of nutrition have significant clinical value in modern medicine.

Biomarkers can be used to assess nutritional status.

There is no clear consensus on best biomarkers for specific nutrients that is applicable for all scenarios.

The application of nutritional biomarkers is actively being investigated.

This chapter lists the most up-to-date resources relevant to the use of biomarkers of nutrition.

Table 2 Professional societies relevant to biomarkers or nutrition

Society name	Web address
Academy of Nutrition and Dietetics	https://www.eatrightpro.org/
African Nutrition Society	https://www.ansnet.org
American Nutrition Association	https://theana.org/
American Society for Nutrition	https://nutrition.org/
American Society of Parenteral and Enteral Nutrition (ASPEN)	https://www.nutritioncare.org/
Australasian Society for Parenteral Nutrition	www.auspen.org.au
British Dietetic Association	https://www.bda.uk.com/
Canadian Nutrition Society	www.cns-scn.ca
Chinese Nutrition Society	https://www.cnsoc.org/
Czech Society for Nutrition	https://www.vyzivaspol.cz/
Danish Nutrition Society	https://www.sfe.dk/dansk1
European Association for the Study of Obesity	https://easo.org/
European Atherosclerosis Society	https://www.eas-society.org/
European Society for Clinical Nutrition and Metabolism	https://www.espen.org/
Federation of European Nutrition Societies	https://fensnutrition.org/
Federation of European Societies on Trace Elements and Minerals (FESTEM)	http://festem.eu/
German Society for Nutritional Medicine	www.dgem.de
Hellenic Society of Clinical Nutrition and Metabolism (GrESPEN)	www.grespen.org
Hong Kong Nutrition Association	https://www.hkna.org.hk/
Indian Council of Medical Research (ICMR)-National Institute of Nutrition (NIN), India	https://www.nin.res.in/
International American Association of Clinical Nutritionists	https://www.iaacn.org
International Atherosclerosis Society	https://www.athero.org/
International Society for Trace Element Research in Humans (ISTERH)	https://www.isterhgroup.org/
International Society for Zinc Biology (ISZB)	http://www.zinc-net.com/ISZB/
International Society of Nutrigenetics/ Nutrigenomics (ISNN)	https://nutritionandgenetics.org/
International Union of Nutritional Sciences (IUNS)	https://iuns.org/
Irish Society for Clinical Nutrition and Metabolism	www.irspen.ie
Italian Society of Artificial Nutrition and Metabolism (SINPE)	www.sinpe.org
Micronutrient Forum	https://micronutrientforum.org/
Nutrition Society	https://www.nutritionsociety.org/

(continued)

Table 2 (continued)

Society name	Web address
Parenteral and Enteral Nutrition Society of Malaysia	http://pensma.my
Society on Sarcopenia, Cachexia and Wasting Disorders	https://society-scwd.org/
Swiss Society for Nutrition	https://www.sfkn.se/
The Society for Nutrition Research [Danish Nutrition Society]	https://www.sfe.dk/dansk1
Turkish Physiological Sciences Association	https://www.tfbd.org.tr
Turkish Society of Pediatric Gastroenterology Hepatology and Nutrition	www.pedgastro.org

This table lists the professional societies involved with biomarkers or nutrition. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers of nutrition within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 1

Table 3 Books on biomarkers or nutrition

Book Title	Authors or Editors	Publisher	Year of Publication
Advances in the Assessment of Dietary Intake	Schoeller DA, Westerterp-Plantenga MS	CRC Press, Taylor and Francis Group	2017
Alzheimer: 100 Years and Beyond	Jucker M, Beyreuther K, Haass C, Nitsch R	Springer	2006
Clinical Aspects of Natural and Added Phosphorus in Foods	Gutiérrez OM, Kalantar-Zadeh K, Mehrotra R	Springer	2017
Diet and Nutrition in Critical Care	Rajendram R, Preedy VR, Patel VB	Springer	2015
Diet and Nutrition in Dementia and Cognitive Decline	Martin CR, Preedy VR.	ScienceDirect Publishing	2015
Diet, Immunity and Inflammation	Calder PC, Yaqoob P	Elsevier Science	2016
Diet, Nutrition and Fetal Programming.	Rajendram R, Preedy VR, Patel VB.	Humana Press, Springer	2017
Endocrine FGFs and Klotho	Kuro-o M	Springer	2012
Foods that Fight Inflammation	Havard Medical School	Harvard Health Publishing	2021
Gout	Newcombe D	Springer	2013
Intracellular Traffic and Neurodegenerative Disorders	George-Hyslop P, Mobley W	Springer	2009
Metabolism and Pathophysiology of Bariatric Surgery. Nutrition, Procedures, Outcomes and Adverse Effects	Preedy VR, Rajendram R, Martin CR	Academic Press	2016

(continued)

Table 3 (continued)

Book Title	Authors or Editors	Publisher	Year of Publication
Molecular Basis and Emerging Strategies for Anti-aging Interventions	Rizvi SI, Çakatay U	Springer	2018
Nutrition and Diet in Maternal Diabetes. An Evidence-Based Approach.	Rajendram R, Preedy VR, Patel VB.	Springer	2018
Nutrition: An Old Disease, a New Insight	Ahmad S	Springer, New York, NY	2013
Nutrition: From Birth to Old Age	Snedden R.	Heinemann Educational Books	2013
Obesity and Nutrition: New Surgical and Nonsurgical Approaches, Cap 6	Iacomino G, Lauria F, Venezia A, Iannaccone N, Russo P, Siani A	Springer Nature	2020
Obesity: Oxidative Stress and Dietary Antioxidants	Marti A, Aguilera C	Elsevier	2018
Sarcopenia- Research and Clinical Implications	Veronese N, Beaudart C, Sabico S	Springer	2021
Sarcopenia: Age-related Muscle Wasting and Weakness Mechanisms and Treatments	Lynch GS	Springer	2011
Sarcopenia: Molecular Mechanism and Treatment Strategies	Sakuma K	Elsevier	2021
Science of Nutrition	Thompson J, Manore M, Vaughan L	Pearson Education (US)	2019
Telomeres, Diet and Human Disease: Advances and Therapeutic Opportunities	Marti A, Zalba G	CRC Press	2017
The Handbook of Biomarkers	Jain KK	Humana Press	2010
The Vitamins	Combs GF(Jr), McClung JP	Elsevier	2017
Uric Acid in Chronic Kidney Disease	Treviño-Becerra A, Iseki K	Karger	2018
Vitamin D in Chronic Kidney Disease	Urena Torres PA, Cozzolino M, Vervloet M	Springer	2016

This table lists books relevant to diet or nutrition in neurological disorders

Table 4 Techniques and platforms related to biomarkers or nutrition

Organization or company name	Web address
BEST (Biomarkers, EndpointS, and other Tools) Resource	https://www.fdanews.com/ext/resources/files/2020/11-24-20-BEST.pdf?1606261388
Biomarker-Menu -BioAgilytix	https://www.bioagilytix.com/biomarker-menu/
Biomarkers, Genetics and Epigenetics - Understanding Society	https://www.understandingsociety.ac.uk/topic/biomarkers-genetics-and-epigenetics
DIANA-mirPath, University of Thessaly	https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath
DIANA-TarBase v8, University of Thessaly	https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=tarbasev8
McGill research and teaching institute	https://www.mcgill.ca/neuro/
Meta Nutrition	https://www.metnu.com/
miRBase, University of Manchester	https://www.mirbase.org/
MitoPedia (specific page on mitochondrial respiratory states)	https://www.bioblast.at/index.php/MitoPedia:_Respiratory_states

This table lists technologies or platforms relevant to biomarkers or nutrition. Please note, occasionally the location of the websites or web address changes

Table 5 Other resources of interest or relevance for health care professionals or patients related to biomarkers or nutrition

Name of resource or organization	Web address
Bioparadigms	https://www.bioparadigms.org/
British Association for Parenteral and Enteral Nutrition: Assessment and Planning	https://www.bapen.org.uk/81-nutrition-support
European Federation of Pharmaceutical Industries and Associations: Working with Patient Groups	https://www.efpia.eu/relationships-code/patient-organisations/
FDA-NIH Biomarker Working Group	https://www.ncbi.nlm.nih.gov/books/NBK338449/
National Institute of Neurological Disorders and Stroke: Patient Organisations	https://www.ninds.nih.gov/Disorders/Support-Resources/Patient-Organizations
Stance4HEALTH	https://www.stance4health.com/
The Patients Association	https://www.patients-association.org.uk/
WHO International Programme on Chemical Safety Biomarkers and Risk Assessment: Concepts and Principles	https://apps.who.int/iris/bitstream/handle/10665/39037/9241571551-eng.pdf
WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation	https://inchem.org/documents/ehc/ehc/ehc222.htm

This table lists other resources of interest or relevance to biomarkers or nutrition. Please note, occasionally the location of the websites or web address changes

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