Handbook of nutrition and diet in leukemia and blood disease therapy

edited by:
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Preface

Blood diseases include a broad range of nutritional deficiencies, leukemias and genetic mutations, with increased infections. Reduced red blood cell production can lead to nutritional diseases, anemias requiring iron supplementation. Patients feel sick, fatigued and have nausea affecting food intake. In this handbook the influence of nutrition on blood cells and diseases is discussed.

Section 1. Blood nutrition and health: overview

Nutrition plays a key role in the health of blood cells and their functions. Nutrition and food can affect blood cells, their proteins including hemoglobin. Such changes affect functioning of coagulation, as well as the immune cells' production of cytokines and immunoglobulin. Hagiwara describes the nutritional needs of stem cells, a growing area of research, and in addition nutritional therapy to treat ulcers in people with sickle cell anemia, a genetically mediated nutritional deficiency. Beliaev and Allen review the management of other types of anemia. The blood cells interactions affect major organ systems. Božič-Mijovski describes hypercoagulability promoting cardiovascular disease and the available laboratory tests to predict these cardiovascular events.

Section 2. Vitamins in therapy of blood cells

Vitamins affect the production of blood cells. Anemia's effect include a variety of health problems described in two reviews discussing the role of vitamin E, provided in novel fashions and conditions. In addition, a review summarizes the role of vitamin C on scurvy during cancer. Das and Chatterjee further discuss vitamin C deficiency. They describe the actions of supplemental vitamin C on tobacco smoke modulated health in myelodysplastic syndrome.

Section 3. Iron in health of blood and disease prevention

Iron levels are of concern in the health of blood. Supplementation with iron was a major focus. Mitra reviewed the needs of low income people for iron. Then, Rocha approached similar problems in Brazilian children using iron supplemented drinking water. Finally, Julian-Almarcegui *et al.* discussed the use of heme iron in comparison to non-heme iron as a supplement.

Section 4. Foods in blood diseases

Foods are natural and traditional means to prevent anemia and deficiencies. Cavalcante and coauthors describe enteral nutrition to increase glutamine in sepsis patients. Other reviewers

describe taurine and L-carnitine as dietary materials affecting blood cell health and anemia. Finally, Jubelirer reviews a neutropenic diet in cancer therapy.

Section 5. Foods, vitamins and minerals affect leukemia

Leukemia is a broad family of cancers originating in the white blood cells of the bone marrow appearing in the blood. While leukemia, especially in children, can be a curable disease, most adult leukemia is just treatable. Of the more than quarter million new cases in the world each year about 80% will die in older adult patients. Some limited evidence suggests that vegetable intake can reduce risk. Shehzad *et al.* reviews the oxidative stress in leukemia and nutritional methods to treat it. Naushad *et al.* described folate metabolism and genetic variants of leukemia responses. Sanchez and Watson describes the potential roles of tea and coffee in leukemia. Thalassemia and the nutritional needs is reviewed by Fung *et al.* The role of obesity in promoting leukemia is reviewed by Orgel. Finally, Thakur reviews nutrition and nutritional management in anemia.

As with other frequently incurable diseases patients resort to items under their control food, dietary supplements and nutritional therapies. A key goal of this book is to review some of these approaches for efficacy in prolonging life and its quality.

R. Ross Watson and Daruka Mahadevan

Blood nutrition and health: overview

1. Multidisciplinary nutritional support in hematopoietic stem cell transplantation

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Abstract

Hematopoietic stem cell transplantation (HSCT) is an effective therapy for haematological malignancies. However, preconditioning regimens using high-dose chemotherapy with or without irradiation cause severe oral and gastrointestinal mucosal damage, and graft-versus-host disease in allogeneic stem cell transplantation causes diarrhoea, liver dysfunction, and skin damage. These adverse effects hinder oral food intake. Moreover, the patient's energy requirement increases markedly because of infection and accelerated metabolism. Consequently, patients who received HSCT often become malnourished. Total parenteral nutrition is used to prevent malnutrition, although it may cause metabolic adverse events and lead to infection. A nutrition support team (NST) endeavors to prevent and manage nutritional complications, and the major elements of the NST comprise nutritional evaluation, planning of nutritional therapy, intervention, and re-evaluation. Enteral nutrition facilitated by the NST may reduce the risk of metabolic disorders, hepatic damage, and infection. Here we report that a multidisciplinary NST reduced the incidence of transplant-related complications, the duration of hospitalization, and the cost of hospitalization. A multidisciplinary team approach may reduce medical expenses.

Keywords: therapy related toxicity, total parenteral nutrition, enteral nutrition, nutrition support team, cost-benefit analysis

Key facts

- Hematopoietic stem cell transplantation is an effective but high-risk treatment for haematological malignancies.
- Patients undergoing transplantation tend to become malnourished due to therapy-related toxicity.
- Poor nutritional status is associated with a high risk of complications and prolonged hospitalization.
- Enteral nutritional support can reduce the incidence of severe complications and improve outcomes.
- A multidisciplinary team approach to patient care may reduce medical expenses.

Summary points

- Hematopoietic stem cell transplantation (HSCT) using high-dose chemotherapy/irradiation is an ultimate therapy for hematological malignancies.
- Preconditioning regimens cause oral mucositis and gastrointestinal mucosal damage.
- Graft-versus-host disease (GVHD) is an important complication that occurs in 20-40% patients who undergo allogeneic stem cell transplantation.
- Patients treated with HSCT often require total parenteral nutrition (TPN) that often causes hyperglycemia and leads to infection.
- The objectives of a nutrition support team (NST) are to facilitate recovery, to maintain body functions, and to prevent metabolic complications of TPN.
- The NST evaluates, plans, intervenes, and re-evaluates patients undergoing HSCT to maintain adequate nutrition.
- Enteral feeding facilitated by the NST may reduce the incidence of metabolic complications and infection.
- A multidisciplinary team approach to the care of patients undergoing HSCT is effective for improving outcomes and reducing costs.

Abbreviations

ALT	alanine transaminase
AST	aspertate transaminase
ASPEN	American Society for Parenteral and Enteral Nutrition
BMI	Body mass index
ESPEN	European Society for Clinical Nutrition and Metabolism
GVHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplantation
IPS	Idiopathic pulmonary syndrome
MM	Multiple myeloma
NST	Nutrition support team
OGA	Objective global assessment
OMAS	Oral mucositis assessment scale
PDSA	Plan-do-study-act
REE	Resting energy expenditure
SCT	Stem cell transplantation
SGA	Subjective global assessment
TPN	Total parenteral nutrition

1.1 Hematopoietic stem cell transplantation and its complications

HSCT re-establishes haematopoiesis by infusing a patient with hematopoietic stem cells to promote hematopoietic recovery after high-dose chemotherapy, irradiation, or both and to induce an anti-tumour allogeneic immune effect. HSCT is mainly used to treat haematological malignancies such as acute leukemia, malignant lymphoma, MM, and chronic leukemia as well as germinal tumours and osteosarcomas. Further, HSCT is effective for treating non-malignant diseases such as aplastic anaemia and severe immunodeficiency.

High-dose chemotherapy with or without total body irradiation is used as a preconditioning regimen. To eradicate malignant cells, high-dose alkylating agents such as cyclophosphamide, nitrosourea, busulfan, and melphalan as well as agents that target the cell cycle, such as high-dose cytarabine and etoposide, are used. These agents cause oral mucositis, diarrhoea, nausea, vomiting, alopecia, hepatic dysfunction, renal dysfunction, and vascular endothelial cell damage (Smith and Wright-Kanuth, 2001). To eradicate tumour cells, fractionated doses totalling 12 Gy of total body irradiation are included in the preconditioning regimen. Low doses (2-4 Gy) may be used to induce an immunosuppressive effect in patients administered non-myeloablative SCT. Ionizing radiation damages the mucosa of the oropharynx, gastrointestinal tract, skin, and lung (Hill-Kayser *et al.*, 2011).

In allogeneic SCT, immunosuppressive therapy is critical to promote engraftment of donor cells and suppress the onset of acute GVHD. Immunosuppressive therapy comprises calcineurin inhibitors

such as cyclosporine and tacrolimus in combination with methotrexate or mycophenolate mofetil to suppress the residual recipient and infused donor lymphocytes. However, calcineurin inhibitors may cause renal and liver dysfunction. Adequate hydration is required to maintain renal blood flow (Arnaout *et al.*, 2014; Woo *et al.*, 1997). Acute GVHD is the most serious complication of allogeneic transplantation, which occurs in 20-40% of patients 14-100 days after transplantation. The symptoms of acute GVHD usually appear as skin lesions, liver dysfunction, and diarrhoea. In gastrointestinal GVHD, anorexia and watery diarrhoea >2,000 ml/d develop, which require nutritional management of water balance and electrolytes as well as treatment of eating disorders (Smith and Wright-Kanuth, 2001).

High-dose chemotherapy and irradiation may induce endothelial injury in the sinusoids of the hepatic vein and cause liver dysfunction and severe jaundice (Coppell *et al.*, 2010; Wadleigh *et al.*, 2003). In patients with severe liver damage, sufficient energy and protein must be provided, therefore TPN is often required. During the neutropenic periods from transplantation to engraftment, patients are at risk of bacterial infection. In particular, damage to the gastrointestinal mucosa may cause the translocation of enteral bacteria from the intestine to the blood stream. The risk of bacteraemia decreases markedly when the neutrophil count exceeds 500/μl; however, the incidence of fungal, cytomegalovirus, and herpes zoster infections are high even after engraftment. Patients who undergo GVHD and treated with steroids are at a high risk of fungal infection, particularly by *Aspergillus* species (Wingard *et al.*, 2010). Microbial infection increases energy expenditure, and patients therefore require sufficient quantities of carbohydrates and protein. TPN facilitates the administration of adequate amounts of glucose and amino acids; however, TPN often causes hyperglycaemia and may increase the risk of infection. Moreover, it is important to control the blood sugar levels of patients treated with TPN.

In the early phase of post-allogeneic SCT, IPS may develop. IPS is often lethal, although it may be prevented using corticosteroids. Patients who develop IPS require higher dietary levels of energy and protein. Evidence indicates that ablation of a recipient's hematopoietic system is required for the engraftment of donor stem cells. Recent studies show that a non-myeloablative preconditioning regimen with adequate immunosuppression allows engraftment of donor cells and allogeneic immune effector cells. Non-myeloablative allogenic SCT makes it possible to treat elderly patients or those with comorbidities. Moreover, it is clear that it is important to treat such patients with supportive therapy that includes nutritional support.

1.2 What is a nutrition support team?

1.2.1 History of nutrition support

The technology to deliver parenteral nutrition was developed after World War II. In 1968, Dudrick *et al.* (1968) inserted a central venous catheter into beagle puppies and raised them using only parenteral nutrition for 72-256 days. Puppies provided only TPN grew equally compared with puppies fed orally (Dudrick *et al.*, 1968). They also showed that TPN can be applied to

patients. The American Society of Parenteral and Enteral Nutrition and the European Society of Parenteral and Enteral Nutrition were established in 1978 and 1980, respectively. Subsequently, TPN was developed for clinical use. However, complications of TPN emerged, such as metabolic dysfunction and infections. Therefore, TPN must be administered by nutritional specialists, and NSTs were first established in the 1970s in several hospitals (Skoutaskis *et al.*, 1975).

An NST typically comprises a dietitian, pharmacist, nurse, and physician. The NST estimates energy expenditure, protein balance, electrolyte balance, and fat and manages TPN (Pietka *et al.*, 2014; Tucker *et al.*, 2015). Administration of excessive or insufficient amounts of calories is often harmful, and patients require caloric intake sufficient to maintain body weight and organ homeostasis. Protein requirements vary according to stressors, such as trauma, surgery, burns, and infections must be correctly estimated. Lipids are essential for the synthesis of mediators of fat metabolism, such as prostaglandins and leukotrienes that maintain the integrity of cell membranes. Lipid deficiency causes alopecia, fatty liver, and thrombocytopenia. Moreover, hyperlipidaemia induces hypertriglyceridemia and liver dysfunction. Thus, according to the estimation of nutritional requirement, the appropriate administration of nutritional elements is critical.

The NST is administered to patients after major surgery, to critical care patients, and to patients with cancer. In particular, the NST is effective for patients undergoing high-calorie fluid therapy who are at a risk of metabolic dysfunction and infections. Moreover, these patients tend to suffer from poor oral food intake and subsequent malnutrition, and enteral nutrition is recommended to utilize and maintain their digestive function. The objectives of the NST are to facilitate recovery from major surgery, maintain body functions, and prevent metabolic complications through appropriate nutritional evaluation, intervention, and reassessment of the patients' nutritional status.

1.2.2 Nutritional evaluation

Nutritional evaluation determines the patients' nutritional status and screens for cachexia. Precise evaluation facilitates early detection of nutritional risks during treatment and comprises SGA and OGA. SGA is a powerful tool for evaluating nutritional status by interviewing patients about the changes in body weight and the amount of oral food intake compared with that when they were healthy (Da Silva Fink *et al.*, 2014). OGA uses objective data such as current body weight, fat thickness, muscle bulk, energy expenditure measured using indirect calorimetry, and serum chemical data, which estimates the amounts of total protein, albumin, transferrin, prealbumin, retinol binding protein, and markers of renal and/or hepatic function such as blood urea nitrogen, ALT, AST, triglycerides, total cholesterol, and blood sugar (Bozzetti *et al.*, 2009). Thus, SGA and OGA estimates the nutritional status and potential risk of malnutrition. When applied to patients administered HSCT, the NST should evaluate the current nutritional status, therapy-related changes in dietary intake, oral mucositis, protein content, and body temperature.

1.2.3 Planning for nutritional support

The plan for nutritional therapy should be developed according to the results of nutritional assessment. Caloric requirements are calculated according to height, weight, and stressors. HSCT requires 130-150% of REE because of patients' increased energy demand. Further, the optimum protein requirement is 1.5-2.0 g/kg/d (Martin-Salces *et al.*, 2008). There is an argument that administration of lipid increases the risk of infection. However, a prospective study in 512 patients receiving bone marrow transplants revealed that the incidences of bacteraemia and fungaemia were the same between two groups of randomly assigned patients who received 6-8% (low dose) or 25-30% (standard dose) of total daily energy provided as a 20% lipid emulsion (Lenssen *et al.*, 1998). An excess dose or rapid administration of a fat formulation may suppress the function of macrophages and oxygen exchange in the lung (Jeejeebhoy, 2012). The ESPEN guidelines propose an optimal dose of lipid of 1.0-1.5 g/kg/d administered at 0.7-1.5 g/kg/12-24 h (Singer *et al.*, 2009). Thus, a lipid formulation can be administered at a safe rate and amount for patients who undergo bone marrow transplantation. To mitigate excessive stress and hypercatabolic status, it is desirable to improve the nutritional status of candidates before bone marrow transplantation. For example, a poor nutritional status before transplantation is associated with increased length of hospitalization (Horsley *et al.*, 2005).

When the transplantation schedule is established, the plan for nutritional support therapy should be implemented as soon as possible. After the preconditioning regimen starts, poor oral food intake develops frequently in the early phase of transplantation due to nausea, vomiting, oral mucositis, and gastrointestinal dysfunction. Despite eating difficulties, enteral nutrition should be encouraged to maintain digestive function. In the ASPEN and ESPEN guidelines, enteral nutrition is recommended for patients treated with SCT (August and Huhmann, 2009; Bozzetti *et al.*, 2009), and several studies have found that enteral nutrition reduces the incidence of transplant-related complications (Guieze *et al.*, 2014; Seguy *et al.*, 2012). However, because of severe nausea/vomiting and oral mucosal damage, administering complete enteral nutrition is often difficult. In such patients, combining partial parenteral and enteral nutrition may be useful for maintaining the nutritional status and digestive function (Azarnoush *et al.*, 2012).

1.3 Intervention using the nutrition support team

The NST includes planning nutritional treatment and management of enteral and parenteral nutrition as well as evaluation, audit/reassessment, and improvement of the nutritional plan. This process is similar to quality management in the manufacturing industry. After World War II, many engineers and scientists came to Japan to restore the country's manufacturing industries, and they taught Japanese engineers and managers. W. Edwards Deming was one of these teachers who proposed a method of quality management called the Deming cycle, which includes the PDSA cycle, and taught Japanese engineers how to implement the PDSA cycle. Japanese engineers adhered to his theory and applied it to quality management of manufacturing, which markedly improved the quality of products manufactured in Japan, leading to the restoration

of numerous industries. For example, the automobile industry developed to the extent that Japanese automobiles and trucks are now highly valued worldwide. Toyota, the representative automobile company, incorporated the concepts of the Deming cycle into the 'Kaizen' method. Kaizen means continuous improvement, which is based on making regular minor changes to continuously improve productivity, safety, and efficiency while reducing waste. Moreover, Kaizen can be applied to medicine. Several hospitals have adopted the Kaizen method and succeeded in improving the quality of their medical care (Iannettoni *et al.*, 2011; Jacobson *et al.*, 2009).

The Department of Emergency Medicine at Vanderbilt University Medical Center adopted the Kaizen method and made 400 improvements to their system of delivering emergency care (Jacobson *et al.*, 2009). Iannettoni *et al.* (2011) evaluated the processes of esophageal resection in their hospital and analysed the costs of surgery, intensive care, and hospitalization. Using the Kaizen method eliminated variability, standardized care, improved patients' outcomes, decreased the length of hospitalization, and reduced therapy-related complications (Iannettoni *et al.*, 2011). The PDSA cycle and the Kaizen method can also be applied to nutritional support. For example, Marcellus *et al.* used the PDSA cycle to implement and evaluate a stepwise oral feeding guideline for infants, with emphasis on parent and care-provider satisfaction, and concluded that a PDSA cycle approach can be used effectively in implementing guidelines and conducting evaluations by a team of multidisciplinary health care professionals (Marcellus *et al.*, 2012).

1.3.1 Nutrition support team for autologous stem cell transplantation

Autologous SCT aims to eradicate tumour cells, although high-dose chemotherapy is employed, because autologous SCT lacks an immune response to tumour cells. Although there is no risk of GVHD, oral and gastrointestinal mucosal damage frequently develop due to strong preconditioning regimens. Such mucosal impairments increase the risk of infection, duration of TPN, and length of hospitalization. Therapy-related mucosal toxicity increases transplant-related mortality and expenses. Using the OMAS, Sonis *et al.* (2001) evaluated the severity of oral mucositis and studied the correlations among OMAS, clinical outcomes, and economics. They found that the OMAS score correlates significantly with the occurrence of infection, days of TPN, days of injectable narcotic therapy, days of hospitalization (>60 days), total hospital charges for the initial admission, and patients' vital status at 100 days (Sonis *et al.*, 2001). The role of the NST in autologous SCT is to prevent and minimize the poor nutrition caused by therapy-related nausea, vomiting, oral mucositis, and diarrhoea. In particular, the NST evaluates nutritional status before and during transplantation as well as the maintenance of adequate enteral nutrition with or without parenteral nutrition. Moreover, mouthwash together with normal saline and oral cryotherapy may be useful for preventing oral mucosal damage.

A retrospective cohort study was conducted to analyse the efficacy of oral cryotherapy or room-temperature saline rinses for preventing oral mucositis in patients with MM or lymphoid malignancies who underwent autologous SCT at a single centre. The study found that oral cryotherapy is more effective than saline rinses to prevent oral mucositis in patients with lymphoma and myeloma receiving conditioning regimens with high-dose melphalan for

autologous SCT (Batlle *et al.*, 2014). Recent studies have shown that proper oral care reduces the incidence of oral mucositis (Bhatt *et al.*, 2010; Yamagata *et al.*, 2012). In a prospective study, the incidence and severity of oral mucositis was compared between 24 patients who received appropriate oral management during transplantation and 24 patients who did not. Oral mucositis was observed in 14 (58.3%) and 22 (91.6%) treated and untreated patients, respectively, and the severity of oral mucositis was lower in the former compared with the latter patients (Yamagata *et al.*, 2012). Therefore, a multidisciplinary NST should cooperate with the oral care team to prevent the deterioration of the nutritional status in patients who undergo SCT.

Rehabilitation provides effective supportive therapy. For example, therapy for dysphagia is required for patients who have difficulty swallowing due to severe mucositis. Aerobic exercises prevent the deterioration of physical performance and reduce the length of hospitalization (Dimeo *et al.*, 1997; Wiskemann *et al.*, 2014). A prospective randomized study compared patients who underwent autologous SCT and participated in an exercise program during hospitalization with patients who did not train. The decrement in performance during hospitalization was 27% higher in the control compared with the training group (P=0.05), resulting in significantly higher maximal physical performance at discharge in the trained patients (P=0.04). Further, the duration of neutropenia (P=0.01) and duration of thrombopenia (P=0.06), severity of diarrhoea (P=0.04), severity of pain (P=0.01), and duration of hospitalization (P=0.03) were reduced by the training group (Dimeo *et al.*, 1997).

The activities of the NST should be linked with the oral care and rehabilitation teams to maximize the effect of each type of specialized supportive care. This will possibly improve the outcomes of patients who undergo HSCT.

1.3.2 Role of the nutrition support team in allogeneic stem cell transplantation

Allogeneic SCT replaces the patient's hematopoietic and immune cell compartments with those of a donor. High-dose radio chemotherapy is used for patients who receive allogeneic SCT, similar to that for patients who receive autologous SCT, and the incidence of oral mucositis and gastrointestinal mucosal damage is quite high for the former patients. Furthermore, GVHD, which is a specific complication of allogeneic SCT, often develops after bone marrow engraftment. Acute GVHD causes fever, liver dysfunction, and gastrointestinal damage. Patients who develop acute GVHD tend to experience eating disorders and malnutrition.

In allogeneic SCT, the objective of the NST is to maintain enteral intake and manage TPN. The NST should propose a meal plan according to the patient's condition and encourage patients to eat by mouth. Appropriate enteral nutrition may reduce the risk of gastrointestinal GVHD-related complications. For example, a retrospective study of 231 patients who received allogeneic SCT showed that the number of days without oral dietary intake correlated with the incidence of acute GVHD grades III-IV; multivariate analysis revealed that >9 days without oral intake was associated with acute GVHD grades III-IV (odds ratio 7.66; 95% confidence interval, 1.44-40.7; P=0.016) (Mattsson *et al.*, 2006). Imataki *et al.* (2006) prospectively compared a cohort of

18 patients with acute gastrointestinal GVHD who received an enteral diet with a cohort of 17 patients who did not. None of the patients who received enteral nutrition developed significant adverse events, including exacerbation of gastrointestinal symptoms. Further, there was no statistically significant difference in the volume or frequency of diarrhoea or the time to complete dietary recovery. Body weight, serum levels of total protein, and albumin improved faster in the enteral nutrition group (Imataki *et al.*, 2006). A retrospective cohort study of 112 patients treated with myeloablative allogeneic SCT found that hyperglycemia is significantly associated with an increased risk of organ dysfunction, acute GVHD grades II-IV, and non-relapse mortality (Fuji *et al.*, 2007).

Nutritional screening and the planning for nutritional intervention are important to ensure the success of allogeneic SCT. First, according to the results of SGA and OGA, the required total energy is calculated. Second, the NST decides on the appropriate balance of carbohydrates, lipids, proteins, vitamins, and trace elements. If poor nutrition or other nutritional problems are detected, nutritional intervention are applied before SCT. In a study conducted using the data of the Japanese Data Center for Hematopoietic Cell Transplantation, 12,050 patients who received allogeneic SCT were stratified according to patients' BMI as follows: <18.5 kg/m^2 (underweight), 18.5-25 kg/m^2 (normal), 25-30 kg/m^2 (overweight), and ≥30 kg/m^2 (obese). Multivariate analysis showed that the risk of non-relapse mortality was significantly higher in the overweight and obese groups compared with the normal group; in contrast, overall survival was shorter in the underweight group (Fuji *et al.*, 2007). Thus, the pretransplantation nutritional status is quite important. Therefore, the NST should act as soon as possible when the schedule of transplantation is established.

During transplantation, patients' characteristics such as body temperature, blood pressure, body weight, and biochemical parameters including albumin, AST, ALT, blood urea nitrogen, blood sugar, and amino acid balance should be monitored to prevent adverse metabolic events. The NST should encourage patients to maintain oral food intake. Patients who develop severe mucositis can be administered total or partial parenteral nutrition with enteral nutrition. Patients treated with allogeneic SCT tend to lose trace elements and vitamins due to the stress of preconditioning regimen, acute GVHD, as well as oral and gastrointestinal mucosal damage. Nannya *et al.* (2014) measured the blood levels of vitamins and trace elements of 15 patients receiving HSCT and found that most patients with deficient levels of vitamins B1 and C as well as zinc develop diarrhoea (Nannya *et al.*, 2014). Therefore, adequate amounts of trace elements and vitamins should be administered. The results of the studies cited above lead us to conclude that collaboration of the NST with the oral care and rehabilitation teams is as important for allogeneic SCT as it is for autologous SCT.

1.4 A study on the multidisciplinary nutrition support team and the effects on medical costs

The multidisciplinary NST of the National Center for Global Health Hospital, Tokyo, Japan (Hagiwara *et al.*, 2011), commenced its activities in 2005. The NST comprises three teams focused on haemato-oncology/infection, chest/endocrinology/neurology, and surgery, respectively. The members include a physician, nurse, pharmacist, and dietitian. Each team conducts rounds twice weekly, and a grand round including the three teams is held twice monthly to discuss intervention for difficult cases. In each ward, link nurses screen for malnutrition of patients and act as mediator between NSTs. Moreover, wound/ostomy/continence nurses collaborate with link nurses and NSTs to determine the most effective nutritional therapies for patients with wounds, decubitus, and skin ulcer.

Before 2005, physicians-in-charge conducted nutritional evaluations and formulated plans for nutritional therapy provided to patients undergoing autologous SCT. Most patients received TPN from post-transplantation days 0, with or without a lipid, and total energy expenditure was estimated as 30-35 kcal/kg/d. Since the multidisciplinary NST commenced operations, all patients undergoing autologous SCT were evaluated for their nutritional status, and plans were made for their nutritional therapy based on the data. A haemato-oncologist led the NST, which included a nurse, dietitian, pharmacist, and dental hygienist. A round of visit to the patients and a team meeting were held twice weekly.

The pretransplantation nutritional status of patients receiving intervention by the NST was evaluated by measuring REE, thickness of the triceps skinfold, and circumference of the arm muscle. REE was measured by a dietitian using an indirect portable calorimeter (Metavine; Vine, Tokyo, Japan). The patients were monitored weekly to evaluate their oral food intake, calories administered through parenteral nutrition, and biochemical parameters such as prealbumin, transferrin, and retinol-binding protein. The total energy expenditure and protein requirements of patients were estimated as 140% of REE and 1.5 g/kg/d, respectively. Nutritional goals were formulated to meet each patient's energy and protein requirements during transplantation. The patients were encouraged to maintain oral food intake as long as possible. TPN administered through a central venous catheter or an amino acid/dextrose solution administered through a peripheral vein with a lipid compound was used in combination with oral nutrition for patients who developed oral mucositis and loss of appetite. Instructions were given to facilitate oral food intake for patients, and consumption of high-calorie liquid foods and protein-containing jellies was advised. The central venous catheter was removed as soon as possible when the patients recommenced oral nutrition. In addition to these nutritional instructions, ambulation in a clean area and exercise using an ergometer were recommended. The dental hygienist evaluated patients' oral conditions and taught them to administer self-care.

The outcomes of 169 cases of autologous SCT were monitored and compared before and after the commencement of intervention by the NST. The median hospital stay before NST was 34.2±12.0 days, which decreased to 26.2±14.8 days (P<0.001) after NST commenced. The median duration of

TPN decreased from 18.4±8.3 to 7.0±7.0 days (P<0.001), and the median number of days without oral food intake improved significantly from 12.6±8.2 to 2.9±3.6 days (P<0.001). Furthermore, the median duration of therapeutic antibiotic use decreased from 14.5±9.8 to 9.9±8.3 days (P=0.001). Liver dysfunction was experienced by 49.3% patients before NST, which reduced significantly to 18.6% after the start of the NST's intervention. Similarly, hyperglycemia reduced from 73.1 to 42.2%, and the incidence of catheter-related infection decreased from 25.4 to 11.8%.

Next, to evaluate whether multidisciplinary NST intervention is cost effective, the cost of parenteral nutrition, therapeutic antibiotics, provision of meals including supplemental food, personnel expenses, and total cost of admission were compared before and after NST commenced. Although there were no significant differences in the cost of parenteral nutrition, antibiotics, and provision of meals, the total cost of hospitalization was decreased significantly by 4,484.40 US$ (P<0.001) per patient. The average length of NST intervention was 89 minutes per patient during hospitalization, and the average personnel cost of one admission was 126.8 US$.

Intervention by a multidisciplinary NST reduced transplant-related toxicities and the duration of hospitalization. Poor nutrition of patients receiving HSCT is associated with increased length of hospitalization (Horsley *et al.*, 2005). Although TPN is useful for preventing malnutrition of transplant patients, a prospective randomized clinical trial revealed that TPN is costlier than individualized enteral feeding (Szeluga *et al.*, 1987). Enteral feeding encouraged by nutritional support can reduce the incidence of transplant-related complications and the duration of hospitalization (Azarnoush *et al.*, 2012; Guieze *et al.*, 2014; Imataki *et al.*, 2006; Murray and Pindoria, 2009).

However, there are arguments about whether a multidisciplinary team is cost-effective (Fleissig *et al.*, 2006; Taylor and Green, 2012), and several studies have addressed this issue. For example, a retrospective analysis of the effect of an enteral nutrition support clinic showed improved quality and reduction in hospital readmissions, tube-related complications, and health care costs (Hall *et al.*, 2014) A study on the cooperation among physicians and pharmacists showed cost effectiveness in home parenteral nutrition (Pietka *et al.*, 2014). In the field of heart failure care, a randomized controlled trial showed that the heart failure program managed by a multidisciplinary team reduces mortality and costs (Capomolla *et al.*, 2002). Thus, a multidisciplinary team is effective in various settings; however, further studies must focus on cost effectiveness.

1.5 Conclusion

HSCT is an ultimate therapy for hematopoietic malignancies, although the incidence of severe complications is high. It may cause the deterioration of a patient's condition and prolong the duration of hospitalization. However, appropriate nutritional support can reduce the incidence and severity of the adverse events of SCT. Intervention by a multidisciplinary team that includes dietitians, pharmacists, physicians, nurses, oral hygienists, and physical therapists may reduce medical expenses.

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2. Measuring hypercoagulability as a risk factor for cardiovascular diseases

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Abstract

Cardiovascular diseases are the leading cause of death globally, resulting in more deaths annually than from any other cause. Cardiovascular diseases are a group of disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and venous thromboembolism. Recognition of the central role of thrombosis in the pathogenesis of cardiovascular diseases has prompted growing interest in the association of hypercoagulability with these diseases. While we can detect hypocoagulability quite reliably using conventional coagulation assay that are widely available, detection of hypercoagulability is more difficult and complex. This manuscript provides a description of the available laboratory tests for measuring hypercoagulability and the existing evidence of the prognostic value of these tests to predict future cardiovascular events.

Keywords: coagulation, thrombophilia, global hemostasis assays

Key facts

- Cardiovascular diseases are the leading cause of death globally: more people die annually from cardiovascular diseases than from any other cause.
- Thrombophilia testing is routinely used for assessing hypercoagulability, but only identifies specific hemostatic disturbances.
- Global assays, such as thrombin generation, overall hemostatic potential or thromboelastometry, aim to capture the whole hemostatic phenotype.

Summary points

- Screening coagulation assays prothrombin time, activated partial thromboplastin time and thrombin time are not useful for determination of hypercoagulability.
- Thrombophilia testing, a panel of assays which usually includes antithrombin, protein C, protein S, activated protein C resistance, prothrombin polymorphism G20210A and antiphospholipid antibodies, is routinely used for assessing hypercoagulability.
- Thrombophilia testing identifies specific hemostatic disturbances, but only the most prothrombotic ones, such as protein C deficiency, or the most common ones, such as resistance to activated protein C.
- Several thrombophilic factors that are either mildly prothrombotic or only become prothrombotic in conjunction with other thrombophilic factors are known, but are currently not routinely determined.
- Markers of coagulation activation can add to thrombophilia testing in detecting hypercoagulability in patients at risk for cardiovascular events.
- Global coagulation assays are available that aim to capture the whole hemostatic phenotype. These are based on measuring thrombin generation, clot formation and degradation or clot viscoelasticity.

Abbreviations

APA	Antiphospholipid antibodies
APC	Activated protein C
APTT	Activated partial thromboplastin time
ELISA	Enzyme-linked immunosorbent assay
F1+2	Fragment 1 and 2
FVL	Factor V Leiden
MI	Myocardial infarction
OCP	Overall coagulation potential
OFP	Overall fibrinolytic potential
OHP	Overall haemostasis potential
PAD	Peripheral artery disease
PCR	Polymerase chain reaction
PT	Prothrombin time
TAT	Thrombin-antithrombin
TE	Thromboelastometry
TG	Thrombin generation
VTE	Venous thromboembolism

2.1 Screening coagulation assays

Screening coagulation assays include PT, APTT and thrombin time. They are routinely used to detect hypocoagulability or for monitoring anticoagulant therapy. Value of these assays to indicate hypercoagulability is less known. PT and thrombin time seem not to detect hypercoagulability, while shorter APTT was consistently found in patients with VTE compared to healthy subjects in several studies and independently of factor VIII levels (Aboud and Ma, 2001; Anzej Doma *et al.*, 2013; Tripodi *et al.*, 2004). Furthermore, patients with shorter APTT were shown to be at an increased risk of VTE recurrence (Hron *et al.*, 2006; Legnani *et al.*, 2006). Shorter APTT was also found in patients hospitalized due to acute coronary event (Abdullah *et al.*, 2010) and in patients with increased in-hospital mortality (Ten Boekel *et al.*, 2003).

Despite being highly statistically significant, the absolute APTT difference between patients and healthy subjects is small (10% or less), making it less useful in an individual assessment. It is also possible that hypercoagulability cannot be detected with all the APTT reagents. A wide variety of APTT reagents are available on the market with substantially different sensitivities towards individual coagulation factor activities (Toulon *et al.*, 1998).

2.2 Thrombophilia

In today's clinical practice hypercoagulability is assessed with thrombophilia testing in patients after an acute thrombotic event. Thrombophilia is defined as an inherited or acquired tendency to develop thrombosis. Routine thrombophilia testing is usually limited to those thrombophilic defects that carry a strong to moderate risk of thrombosis: antithrombin, protein C and protein S deficiencies, FVL, prothrombin G20210A polymorphism and APA (Stegnar, 2010).

2.2.1 Antithrombin

Antithrombin is the most potent natural inhibitor of thrombin, factor Xa, factor IXa, and also factors XIa, XIIa and kallikrein. Congenital antithrombin deficiency is a rare autosomal dominant disorder. The majority of affected individuals are heterozygotes, whilst homozygous mutations are mostly lethal. The prevalence of antithrombin deficiency in general population is about 0.02% (Tait *et al.*, 1994). Acquired antithrombin deficiency may occur in a variety of clinical settings, such as disseminated intravascular coagulation, nephrotic syndrome, metastatic tumors, etc. In chronic liver disease antithrombin levels decrease due to reduced synthesis, but an increased risk of thrombosis does not follow (Bick and Ucar, 1992). Congenital antithrombin deficiency can be quantitative (type I) or qualitative (type II) with reduced functional but normal immunological levels of antithrombin (Pineo and Hull, 1998).

Type I deficiency is the most prevalent among patients with thrombosis, while type II appears to be less thrombogenic (Patnaik and Moll, 2008). The prevalence of any type of antithrombin deficiency is about 1% of consecutive patients with a first VTE (Pineo and Hull, 1998). Antithrombin deficiency is also associated with MI (Roldan *et al.*, 2009) and stroke (Puurunen *et al.*, 2013).

The recommended assay for detection of antithrombin deficiency is a functional assay, because it can detect both quantitative and qualitative deficiencies. Antigen measurements should not be used to screen patients, but only for further characterization of defects identified by one or more functional assays (Tripodi and Mannucci, 2001). Functional assays do not, however, detect all clinically relevant antithrombin defects. These can only be detected with gene analysis (Fischer *et al.*, 2013).

Normal plasma antithrombin levels range between 75-120%. Although there are some variations in normal ranges, such as slightly lower levels in women taking oral contraceptives and elderly men, these variations are mild. Most patients with inherited heterozygous deficiency have levels in the range of 40-60%. Lower levels within the reference range of antithrombin activity has been observed in women who suffered VTE during oral contraceptive usage long after oral contraceptives discontinuation (Anzej Doma *et al.*, 2013). Whether low normal antithrombin predisposes these women to increased rate of recurrent VTE is not known. Low normal antithrombin was associated with elevated risk for recurrent cardiovascular events and shorter event-free time in acute coronary syndrome patients (Pelkonen *et al.*, 2005), and with PAD with higher incidence of antithrombin deficiency in patients with critical limb ischemia than patients

with claudication (Komai and Juri, 2009). However, in the Atherosclerosis Risk in Communities Study (ARIC), that recruited 14,477 adults aged from 45 to 64 years who were initially free of coronary heart disease, no association between antithrombin levels and coronary disease risk after 5-year follow-up was found (Folsom *et al.*, 1997).

2.2.2 Protein C

Protein C is a vitamin K-dependent coagulation protein that inhibits the coagulation system primarily through inactivation of factors V and VIII, the cofactors required for activation of thrombin and factor Xa. Protein C deficiency is rare, occurring in general population at approximately 0.2%. Quantitative deficiency (type I) is the most common form, with protein C levels of about 50%. In qualitative deficiency (type II) protein C levels are normal, but functional levels are reduced. Acquired deficiency is detected in disseminated intravascular coagulation, in the presence of acute VTE, in severe liver disease, in hemolytic uremic syndrome and in thrombotic thrombocytopenic purpura (Baker and Bick, 1999).

For detection of protein C deficiency functional laboratory assays should be used. Antigen measurements can only serve for further characterization of the defect (Tripodi and Mannucci, 2001). In a functional assay, protein C must first be activated by either snake venom, thrombin or thrombin-thrombomodulin complex. APC can then be detected with the use of a specific chromogenic substrate or by the formation of fibrin clot. Functional assays, however, cannot detect all deficiencies; Protein C Padua 2 is a rare prothrombotic genetic variant that is undetectable by functional assays (Girolami *et al.*, 1993), but is detectable by clot-based assays. Another rare genetic variant (Asn2Ile substitution) is detected by neither (Cooper *et al.*, 2011).

Reduced protein C activity was reported in young patients with MI (Zorio *et al.*, 2006). Low normal level of protein C was shown to increase the risk for recurrent cardiovascular events (Pelkonen *et al.*, 2005) and stroke (Lu *et al.*, 2013). The arterial event free rate of PAD patients with protein C deficiency was significantly lower than in patients without the deficiency during the two-year follow-up (Komai and Juri, 2009). Patients with coronary artery disease and decreased protein C were show to be at increased risk of sudden death (Lauribe *et al.*, 1992).

2.2.3 Protein S

Protein S is a cofactor for the protein C-induced inactivation of factor V and VIII. About 60% of protein S in plasma is bound to C4b binding protein, whereas 40% is free and active as APC cofactor (Dahlback, 1995). Congenital protein S deficiency is an autosomal dominant disorder and is found in 1-3% of patients presented with a first episode of VTE (Pineo and Hull, 1998). Protein S deficiency is a heterogeneous group of disorders with three separate types (Table 2.1). Type I and type III deficiencies account for 95% of cases of protein S deficiency (Wypasek and Undas, 2013). Homozygous protein S deficiency is rare, but can be present as neonatal purpura fulminans, reflecting severe disseminated intravascular coagulation caused by the absence or near absence of plasma protein S. Acquired protein S deficiency can develop with vitamin K

Table 2.1. Types of protein S deficiency.

	Total protein S	Free protein S	Protein S activity
Type I	decreased	decreased	decreased
Type II	normal	normal	decreased
Type III	normal	decreased	decreased

deficiency, treatment with vitamin K antagonists, liver disease, severe and chronic inflammation, autoimmune syndromes, nephritic syndrome or disseminated intravascular coagulation (Mulder *et al.*, 2011). Low protein S levels are commonly observed during pregnancy.

Total and free protein S can be measured with ELISA. Measurement of the total antigen requires incubation times long enough to allow complexed protein S to be released from C4b binding protein and to react quantitatively with anti-protein S antibodies bound to the plate. Measurement of the free antigen requires either pretreatment of test samples to separate the free from the bound antigen before reaction with the antibody or the use of a monoclonal antibody that recognizes only the free form of protein S (Tripodi and Mannucci, 2001). In functional protein S activity assays, the effect of free protein S as a cofactor to APC is determined. These assays are predominantly coagulometric and measure the prolongation of the clotting time due to free protein S activity in the degradation of factor Va and factor VIIIa by APC. An assay of the APC-independent anticoagulant activity of protein S with improved resolution has been developed in which the clotting time is determined in the presence and absence of a polyclonal protein S antibody (Van Wijnen *et al.*, 1998). Since functional tests are influenced by several pre-analytical and analytical factors, immunological methods are preferred. Free protein S antigen distinguishes carriers of protein S deficiency from non-carriers much better that total protein S, therefore, free protein S determination is usually included in the routine thrombophilia testing (Simmonds *et al.*, 1998).

Patients with low protein S levels and known mutation within PROS1 gene are at increased risk of recurrent VTE and require lifelong oral anticoagulation (Wypasek and Undas, 2013). Free protein S level can identify young subjects at risk for VTE in thrombophilic families (Lijfering *et al.*, 2009). Compared with non-deficient family members, subjects with protein S deficiency have a higher risk for atherothrombotic event before 55 years of age that is independent of prior VTE (Mahmoodi *et al.*, 2008). On the contrary, the Second Northwick Park Heart Study reported an association between high free protein S coronary heart disease patients, but not with stroke (Ken-Dror *et al.*, 2011).

2.2.4 APC resistance and Factor V Leiden

APC is a key anticoagulant enzyme needed for the proper down-regulation of blood coagulation. Reduced anticoagulant response to APC (APC resistance) is a major risk factor for VTE in Caucasians. In more than 95% of cases the APC resistance is caused by single nucleotide polymorphism in the coagulation factor V gene (FVL). FVL is partially resistant to inactivation by APC, which allows for longer duration of TG and leads to a hypercoagulability. It is inherited as an autosomal dominant trait and has a prevalence of 3-8% in the general population and about 25% in patients with VTE (Bedencic *et al.*, 2008). Two other gene variants in coagulation factor V were described: factor V Cambridge (Williamson *et al.*, 1998) and factor V Hong Kong (Chan *et al.*, 1998), but they yield only slightly decreased APC cofactor activities (Norstrom *et al.*, 2002).

APC resistance is most commonly assessed with APTT-based methods with and without the addition of APC. These methods are simple and inexpensive. If the test plasma is pre-diluted with factor V deficient plasma the methods become close to 100% sensitive and specific for FVL (Tripodi and Mannucci, 2001). Another sensitive and specific functional assay is based on the incubation of the test plasma with factor V depleted plasma, APC and Russell's viper venom. Reagent that contains factor V-dependent prothrombin activator Noscarin from the venom of *Notechis scutatus scutatus* and EDTA are then added and the clotting time recorded. If the activated factor V molecules in the sample have been eliminated during the incubation step, the velocity of prothrombin activation by the FVL-dependent Noscarin is slow, and, therefore, the clotting time is long. If the activated factor V elimination has been incomplete (eg, due to FVL), the velocity of prothrombin activation is high and the clotting time is short (Wilmer *et al.*, 2004). Despite high sensitivity and specificity of the functional methods APC resistance is usually confirmed with a DNA-based method. Several DNA-based methods are sufficiently sensitive, robust and accurate. They are usually based on PCR for quantification of the DNA sequence of interest. Detection of the FVL is best performed using real-time PCR analysis as this relatively simple technique allows its discrimination from rare variants of neighboring nucleotides (Cooper *et al.*, 2012), but since this is a rapidly growing field other techniques may become available in the near future.

APC resistance is the most common risk factor for VTE known to date. Not only FVL-dependent APC resistance, but also APC resistance due to other causes is associated with an increased risk of VTE and this association is dose dependent. This may be due to the fact that several well-known risk factors for VTE other than FVL, including elevated levels of prothrombin or coagulation factor VIII, decreased levels of protein S or tissue factor pathway inhibitor, oral contraceptive use, pregnancy and cancer, are associated with a poor response of plasma to APC (Castoldi and Rosing, 2010). APC resistance seems not to be associated with coronary artery disease. One study reported an association between APC resistance and juvenile or recurrent stroke (Halbmayer *et al.*, 1994).

2.2.5 Prothrombin G20210A

The second most prevalent polymorphism linked to thrombophilia is considered to be the guanine to adenine transition at nucleotide position 20210 in the 3'-untranslated region of the prothrombin gene, resulting in an increased plasma level of prothrombin. Similarly as for FVL, several DNA-based methods are available also for prothrombin G20210A genotyping that are highly sensitive and specific.

The prothrombin 20210A allele is associated with a 2- to 3-fold increased risk of VTE. It is found in 2-4% of general population and in 6-16% of patients with VTE (Bedencic *et al.*, 2008) and in 20% of patients with cerebral venous sinus thrombosis, making it the most prevalent thrombophilic marker the latter (Martinelli *et al.*, 1998). The combination of prothrombin G20210A polymorphism with oral contraceptive use increases the risk of cerebral venous sinus thrombosis 10-fold (Martinelli *et al.*, 1998). Prothrombin G20210A was not consistently associated with MI (Ridker *et al.*, 1999; Sode *et al.*, 2013), but was found to be associated with ischemic stroke in young adults (Jiang *et al.*, 2014).

2.2.6 Antiphospholipid antibodies

APAs bind to phospholipid-associated plasma proteins such as β_2-glycoprotein I and prothrombin. They are found in 1-5% of general population. Presence of APA is confirmed when either anti-β_2-glycoprotein I antibodies, anti-cardiolipin antibodies or lupus anticoagulants are detected twice at least 12 weeks apart.

APAs are strongly associated with VTE and arterial thrombosis, and also recurrent miscarriages, heart valve abnormalities, thrombocytopenia, chorea and livedo reticularis. These additional features distinguish APAs from other thrombophilias (De Groot and Derksen, 2005). VTE occurs in up to 55% of patients with APA. Arterial thrombosis is less common. Strokes and transient ischemic attacks account almost half of the arterial occlusions. Other anatomic sites for arterial thrombosis are the heart (coronary occlusion), the eye, kidney and peripheral arteries (Franchini and Veneri, 2005). Although the majority of patients develop venous or arterial thrombosis at a single anatomical site, a minority presents with multi-organ involvement of rapid onset. This dramatic event, also called 'catastrophic' antiphospholipid syndrome is associated with a high mortality as a consequence of multi-organ failure (Asherson and Cervera, 2003).

2.2.7 Other thrombophilic factors

Other thrombophilic factors, such as hyperhomocysteinemia, elevated levels of coagulation factors VIII, IX, XI and fibrinogen, and several genetic polymorphisms (MTHFR 677C/T, PAI-1 4G/5G, FXIII Val34Leu, endothelial protein C receptor 6936A/G, thrombomodulin C1418T, etc.) are either mild thrombophilic factors or only become thrombophilic in conjunction with other thrombophilic factors. They are less useful for determination of hypercoagulability of an individual, but rather investigated in large clinical studies.

2.3 Markers of coagulation activation

Blood coagulation is initiated by the release of tissue factor from activated endothelial and blood cells. A cascade of reactions (coagulation cascade) follows that ends with the formation and subsequent dissolution of fibrin clot. During this process different peptides are cleaved (prothrombin F1+2 from prothrombin, fibrinopeptide A and B from α and β polypeptide chains of fibrinogen) and enzyme-inhibitor complexes (thrombin-antithrombin) are formed that can be utilized as markers of coagulation activation. D-dimer, an end product of fibrin (but not fibrinogen) degradation, is also the result of coagulation and subsequent fibrinolysis activation. All individuals have a measurable amount of activation markers in their circulation, confirming the Astrup's original hypothesis that low grade coagulation and fibrinolysis are continuous processes of wear and tear in normal individuals (Astrup and Permin, 1947). During systemic or even local activation of coagulation and fibrinolysis, increased amounts of activation products are generated. Therefore, measurement of activation markers has been proposed as a method of assessing the hypercoagulable state of an individual (Stegnar *et al.*, 2003/2004). All coagulation activation markers can be measured by ELISA. For D-dimer, the most commonly measured coagulation activation marker, several other immunological techniques exits and are utilized by general medical laboratories.

Prothrombin F1+2, TAT and fibrinopeptides A and B were found to be very sensitive to pre-analytical factors (Herren *et al.*, 1994; Stegnar *et al.*, 2007). In some, but not all studies increased F1+2 and TAT were reported in individuals with different thrombophilic defects. Although higher levels of F1+2 and TAT have been reported in patients with VTE compared to patients without this disease (Božič *et al.*, 2002) and in such hypercoagulable states as a healthy pregnancy (Eichinger *et al.*, 1999), their association with hypercoagulable states remains unclear (Vižintin Cuderman *et al.*, 2011). In the Caerphilly study F1+2 and TAT were evaluated, but neither of them was associated with incident ischemic heart disease (Lowe *et al.*, 2001) nor could Folsom and co-workers (Folsom *et al.*, 2001) establish an association between incident coronary heart disease and F1+2 in baseline plasma samples of subjects who developed coronary heart disease.

At present D-dimer is the most extensively used marker of coagulation activation. It is best known as the biochemical gold standard for an initial assessment of suspected VTE. With high sensitivity and a negative predictive value of nearly 100% it is an integral part of diagnostic algorithms to exclude VTE. In patients with acute VTE, D-dimer is elevated and decreases after 1 to 3 days of treatment, but remains above normal levels for at least the first week of treatment (Peternel *et al.*, 2002; The DVTENOX Study Group, 1993). This is probably due to prolonged fibrinolysis, which is independent of heparin therapy and TG, but it may also be partly due to the relatively long half-life of D-dimer. By the first month of treatment D-dimer levels mostly return to normal and remain so throughout warfarin treatment (Vižintin Cuderman *et al.*, 2011). After discontinuation of anticoagulant treatment, D-dimer levels remain higher in patients with thrombophilia than in those without (Palareti *et al.*, 2003; Vižintin Cuderman *et al.*, 2011). Normal levels of D-dimer one month after cessation of anticoagulant treatment have been shown to have a high negative predictive value for recurrent VTE, while elevated D-dimer significantly increased the risk of

recurrent VTE in patients with unprovoked previous VTE and this risk could be reduced with resumption of anticoagulant therapy (Palareti *et al.*, 2006). D-dimer was also shown to be a strong, consistent predictor of cardiovascular death, MI, stroke, leg ischemia, arterial surgery and post-surgery arterial occlusion in several prospective studies (Stegnar *et al.*, 2003/2004).

2.4 Global coagulation assays

In contrast to thrombophilia testing, which resembles searching for a needle in a haystack, and measuring specific end products of coagulation activation, global coagulation assays aim to capture the whole hemostatic phenotype. These methods are based on measuring: (1) TG; (2) clot formation and degradation; or (3) clot viscoelasticity.

2.4.1 Thrombin generation

The capacity to generate thrombin and the enzymatic action of thrombin determine blood coagulability. Therefore, measurement of TG, and in particular the enzymatic potential of thrombin, provides a method for quantifying the composite effect of the multiple factors that determine coagulation capacity and the influence of the environment on these factors (Baglin, 2005). The magnitude of TG is determined by the concentrations of all the known clotting factors and inhibitors together with some plasma proteins that modulate the response.

Measurement of TG was first described by Macfarlane and Biggs as early as 1953 (Macfarlane and Biggs, 1953). They used whole blood from which at several time points sub-samples were transferred to fibrinogen solution heated to 37 °C and clotting time recorded that corresponded to thrombin concentration. In the last decades, specific thrombin substrates, technologically advanced photometers and computers simplified TG measurement considerably.
Numerous variants of the methodology exist and can be classified as:

- methods with high tissue factor concentration and short reaction times;
- methods with low tissue factor concentration and long reaction times;
- methods starting with tissue factor of contact activation or none and methods that combine tissue factor and contact activation;
- methods using different types and mixtures of lipids in different concentrations.

The most wide spread commercially available methods are the CAT (Calibrated Automated Thrombogram; Diagnostica Stago, Gennevilliers, France) and the TG Assay (Technoclone; Vienna, Austria) (Figure 2.1).

Increased TG was associated with increased risk of a first idiopathic VTE and identified patients at higher risk of VTE recurrence in several studies (Besser *et al.*, 2008; Eichinger *et al.*, 2008; Hron *et al.*, 2006; Tripodi *et al.*, 2008). Increased endogenous thrombin potential and thrombin peak height were found in patients with MI (Smid *et al.*, 2013) and stroke (Rooth *et al.*, 2013).

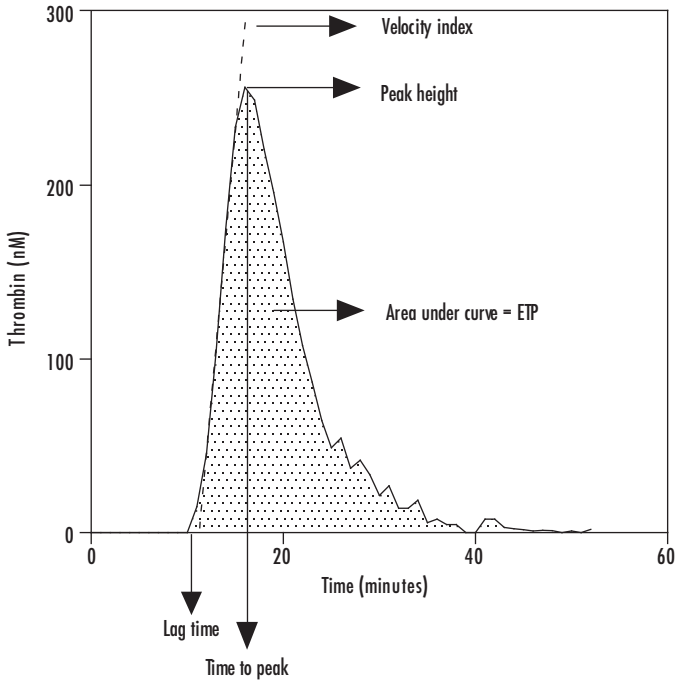

Figure 2.1. Thrombogram is the first derivative of raw data (fluorescence readings) after some mathematical transformations. ETP = endogenous thrombin potential.

2.4.2 Overall hemostasis potential

OHP is based on repeated spectrophotometric registration of fibrin formation in two parallel samples of citrated plasma, to which small amounts of thrombin and t-PA (the first plasma sample) or only thrombin (the second plasma sample) are added. The areas under the fibrin formation curves obtained represent OHP (the first plasma sample with thrombin and t-PA) and OCP, the second plasma sample with only thrombin added). OFP is calculated as the difference between OHP and OCP (Figure 2.2) (He *et al.*, 2001). The OHP assay is not commercially available and it is not yet standardized. It is, however, inexpensive, easy and fast to perform (Antovic, 2008). It offers an insight in fibrinolysis and takes into account fibrinogen, another marker of hypercoagulability, which is not accounted for in TG assays.

Increased OHP has been associated with pregnancy-provoked VTE and was more severe in women with concomitant thrombophilia (Antovic *et al.*, 2003). In another study, OHP also identified hypercoagulable states in patient's antiphospholipid syndrome (Curnow *et al.*, 2007). In young patients increased OHP and decreased OFP were documented long after an acute cerebral ischaemic event (Anzej *et al.*, 2007). Decreased OFP was also documented in stable coronary artery disease patients compared with healthy controls (Reddel *et al.*, 2013).

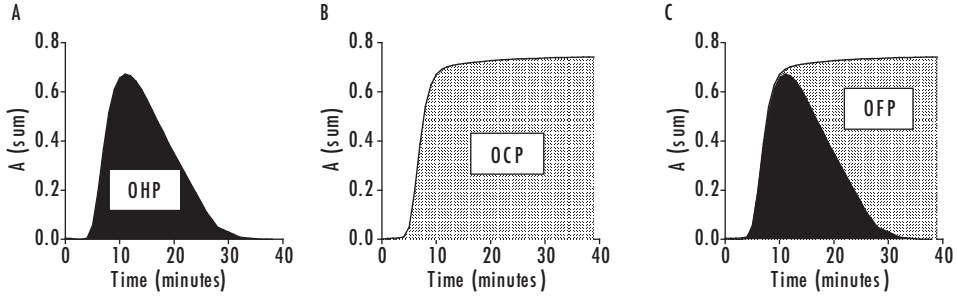

Figure 2.2. (A) Overall haemostasis potential (OHP). (B) Overall coagulation potential (OCP). (C) Overall fibrinolytic potential (OFP).

2.4.3 Thromboelastometry

TE, a viscoelastic method of hemostasis testing, was first described by Hartert (1948). Today two commercially available systems are available: the thrombelastography (TEG®, Hemoscope Corporation, Niles, IL, USA) and the rotational TE (ROTEM®, Tem International GmbH, Munich, Germany). Both TEG® and ROTEM® provide global information on the dynamics of clot development, stabilization and dissolution that reflect *in vivo* haemostasis. TEG® operates by moving a cup in a limited arc (±4°450 every 5 seconds) filled with sample that engages a pin/wire transduction system as clot formation occurs, whereas the ROTEM® has an immobile cup wherein the pin/wire transduction system slowly oscillates (±4°450 every 6 seconds). Several variables of the recording can be evaluated (Table 2.2, Figure 2.3). TEG® and ROTEM® commonly employ citrated whole blood that is re-calcified to initiate coagulation. It is also common to use an activator as this standardizes the test and in addition speeds up the rate at which clotting takes place and hence the rate at which a result is generated.

TE showed a marked hypercoagulable profile in patients with acute VTE (Spiezia *et al.*, 2008). Also in patients with a history of VTE, shorter clotting times and accelerated maximum velocity of clot propagation were measured (Hvitfeldt Poulsen *et al.*, 2006). In individuals with a personal or family history of VTE, hypercoagulable TE was measured in 40% of patients, but it did not correlate with thrombophilic status (O'Donnell *et al.*, 2004). TE therefore cannot be used as a screening test for thrombophilia, but it might be a useful adjunctive test, particularly in patients without known thrombophilic defects. Hypercoagulability detected with TE was shown to predict recurrent ischemic events after stenting in coronary artery disease patients (Gurbel *et al.*, 2009), but no differences in TE parameters were found in patients with history of cerebral vein thrombosis (Koopman *et al.*, 2009). Further studies are needed to determine whether TE can be used to predict the recurrence of thrombotic events.

Table 2.2. Parameters of the TEG® and ROTEM®.

	TEG®	ROTEM®
Measurement period	–	reaction time (RT)
Time from start to when the waveform reaches 2 mm above baseline	R	clotting time (CT)
Time from 2 mm above baseline to 20 mm above baseline	K	clot formation time (CFT)
Alpha angle (°)	a (slope between R and K)	a (angle of tangent at 2 mm amplitude)
Maximum angle	–	CRF
Maximum strength	maximal amplitude (MA)	maximal clot firmness (MCF)
Time to maximum strength	–	MCF-t
Amplitude at a specific time	A30, A60	A5, A10...
Clot elasticity	G	maximum clot elasticity (MCE)
Maximum lysis	–	clot lysis fraction (CLF)
Clot lysis at a specific time (min)	CL30, CL60	LY30, LY45, LY60
Time to lysis	2 mm from maximal amplitude	clot lysis time (CLT; 10% difference from MCF)

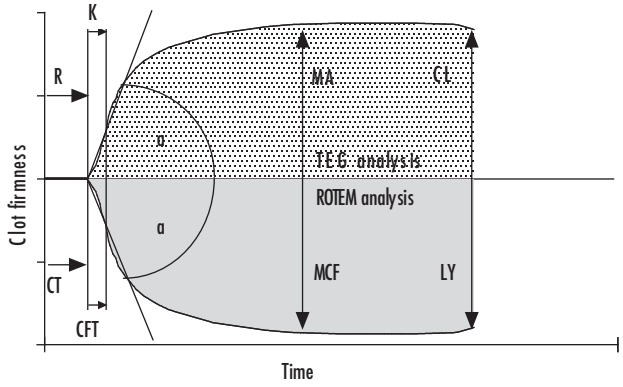

Figure 2.3. Thrombelastograph from TEG® or ROTEM®. CFT: clot formation time; CL: clot lysis; CT: clotting time; K: time from 2 mm above baseline to 20 mm above baseline; MA: maximal amplitude; MCF: maximal clot firmness; LY: clot lysis; R: time from start to when the waveform reaches 2 mm above baseline

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3. Heparin-induced thrombocytopenia

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Abstract

Heparin-induced thrombocytopenia is an antibody-mediated adverse drug reaction, which may associate with thrombosis, paradoxical for an anticoagulant and low platelet count. The underlying pathogenesis is uncontrolled thrombin formation at platelet, monocyte and vascular endothelial surfaces and their microvesicles leading to generalized tendency of thrombosis. Thromboembolic complications may include both venous thrombosis and arterial ischemia, limb necrosis, myocardial infarction and stroke and impairment of other organ functions. The incidence of heparin-induced thrombocytopenia (HIT) is dependent on clinical circumstances, but may vary at 0.01-5%. Unfractionated heparin causes HIT ten-fold more often than low molecular weight heparin. Surgery, perfusion therapies, platelet transfusions and immunological susceptibility enhance the risk and influence the outcome of HIT. Clinical suspicion is assessed by a 4Ts score test but confirmed with laboratory tests. Typically, the exposure for more than 4 days to heparin ('t' of timing), lack of other ('t' of other), causes for thrombocytopenia ('t' of thrombocytopenia), and new or progressing thrombosis ('t' of thrombosis), are clinical landmarks of HIT. During heparin therapy at an estimated HIT risk to exceed 1%, platelet count should be monitored every three days for 4-14 days or until heparin is stopped. Later onset HIT is possible. Upon diagnosis anticoagulation should be rapidly replaced. It is also important to exclude asymptomatic leg vein thrombosis to tailor the dosing of anticoagulation, prophylaxis vs. therapy. In HIT with (HITT) or without thrombosis and normal renal function, argatroban or danaparoid is recommended. In patients with HITT and renal insufficiency, argatroban is the drug of choice, whereas in pregnancy and at liver failure danaparoid is recommended. Further studies on fondaparinux and direct oral anticoagulants in HIT are needed.

Keywords: platelets, thrombin, microparticles, coagulation, thrombosis

Summary points

- Heparin, an important drug designed for prevention and treatment of thrombosis, may as a paradoxical rare adverse effect trigger thrombosis in both veins and arteries by an immunological mechanism.
- The early recognition of sudden decrease of platelet count (thrombocytopenia) in association with heparin treatment and with some established risk factors is highly important.
- Early diagnosis improves patient outcome, as still some 30-40% mortality may be associated with heparin-induced thrombocytopenia (HIT).
- The management should be individually tailored with another anticoagulant, either a heparinoid or a thrombin inhibitor, either to prevent or treat thrombosis.
- As HIT may reoccur upon new administration of heparin patient education and a card or alert in the patient files to warn about the syndrome are key issues.

Key facts

- Heparin therapy whether used prophylacticly or for treatment of thrombosis, may paradoxically turn prothrombotic with an immunological mechanism and induce thrombocytopenia, HIT.
- Overwhelming coagulation activity, generation of microparticles and platelet consumption to vascular surfaces may result in overt thrombosis in either veins or arteries or even both.
- HIT is a rare (0.1-5%) drug-induced adverse reaction, but a high degree of suspicion is warranted among patients receiving heparin and developing sudden thrombocytopenia with or without thrombosis.
- HIT usually appears at 5-10 days after heparin initiation, but a later onset is possible when other causes for thrombocytopenia are absent or unlikely.
- The anticoagulation must be rapidly switched to either argaratroban, bivalirudin or danaparoid and after the acute state to fondaparainux.
- Platelet count should be followed to normalize, usually within a week, and risk of renewal is to be noted later if anticoagulation is to be used again.

Abbreviations

AntiFactor Xa	Antithrombin-based FXa inhibitory activity
APTT	Activated partial thromboplastin time
DIC	Disseminated intravascular coagulation
ELISA	Enzyme-linked immunosorbent assay
FV/VIII/X/Xa	Coagulation factor V/VIII/X/Xa
HIT	Heparin-induced thrombocytopenia
HITT	Heparin-induced thrombocytopenia with thrombosis
INR	International normalized ratio of prothrombin time assessment
LMWH	Low molecular weight heparin
PF4	Platelet factor 4
PT	Prothrombin time
TTP	Thrombotic thrombocytopenic purpura
UFH	Unfractionated heparin

3.1 Heparin, still the mainstay of anticoagulation

Despite the current vast developments of the novel direct oral anticoagulants heparin continues to be a mainstay of anticoagulation. This is true in hospitalized patients, to execute plasma exchange, hemodialysis, hemodiafiltration and perfusion or other kidney replacement therapies and to be able to perform vascular and thoracic surgery and invasive radiological procedures. In many special patient groups and in particular for medical indications of thromboprophylaxis, heparins remain the treatment of choice, also because of the vast experience associated with its use. In cancer patients the prevention and treatment of thrombosis are based on heparin therapies, as both UFH intravenous infusions and subcutaneous administration of LMWH are effective in acute treatment, as well as short- and long-term prevention of thrombosis, respectively. Also, pregnancy-associated thromboprophylaxis or treatment of possible thrombosis is another important indication area. In the future it is likely that the use of heparin will diminish, although UFH during vascular and cardiac surgery is unlikely to be replaced due to its effectiveness and so far the low costs. UFH can be reverted rapidly with protamine at the end of surgery or under hemostatic disturbances.

3.2 Source and mechanism of action of heparins

Heparin is the naturally occurring substance, which is isolated nowadays mainly from porcine gut mucosa and manufactured as the parenteral anticoagulant (Lassila and Jouppila, 2014). Heparin proteoglycans derive from mast cells and they are enzymatically or chemically modified to release the heparin chains and degraded into the targeted entities UFH, having an average molecular weight of 15,000-17,000 Da and smaller entities, i.e. LMWH, having a molecular weight of 3,500-7,500 Da.

Heparin's function is mainly dependent on antithrombin, the naturally occurring anticoagulant, which is produced by liver. There is a critical pentasaccharide sequence in heparins, which is responsible for the main anticoagulant action achieved by binding of antithrombin to heparin. Thereby the action of naturally occurring plasma antithrombin is enhanced by 1000-fold (Bourin and Lindahl, 1993). If the heparin chain is long, having more than 18 pentasaccharides available, thrombin is inhibited, as by UFH, for which the LMWH chains are too short. In contrast, LMWH mainly inhibits FXa, which occurs without the need of triple complex formation, whereas UFH is inhibiting many coagulation factors.

Moreover, all naturally occurring anticoagulants (antithrombin, protein C and protein S, tissue factor pathway inhibitor and heparin cofactor II) benefit from the action of heparin, which enhances their activity to control thrombin generation and inhibit the formed thrombin (Bourin and Lindahl, 1993). It is useful not only to widely control the activity of coagulation factors with heparin, but also to preserve the vitamin K-dependent anticoagulants protein C and S synthesis which are important regulators of coagulation activity. It is critical to ensure the availability of vitamin K in nutrition and improve its absorbance, temporarily when needed by using the parenteral route.

Many times in cancer patients natural anticoagulants are reduced, and thereby also the anticoagulant action of heparin may be impaired. There may also be heparanase-activity in cancer cells which further impair the anticoagulant environment (Nadir and Brenner, 2014). In expert opinion reports it has been suggested that a 25-30% higher LMWH dose can be temporarily used in cancer patients to improve the efficacy, meticulously following the risk for bleeds (Carrier *et al.*, 2014). Of heparins UFH demand daily APTT-based monitoring when administered intravenously, while LMWH can be mainly administered without monitoring, however, in pregnancy, renal insufficiency and at high dosing the AntiFactor Xa activity should be checked from time to time (Hirsh and Ratschke, 2004). AntiFactor Xa activity measurement can help to maintain the adequate level of anticoagulation (Ortel *et al.*, 2015). In cancer patients FVIII and von Willebrand factor are often at a very high level (above 200%) and may further attenuate the effect of heparins.

Furthermore, tissue factor pathway inhibitor, which is not measured by the routine coagulation laboratory, may also at an upregulated level enhance the anticoagulation, in contrast to the low levels, which may associate with uncovered warfarin and heparin 'resistance' (Orfeo *et al.*, 2011).

3.3 Safety of heparin and its side effects

The long term safety of LMWH is confirmed in cancer (Akl *et al.*, 2014), the pregnant patient population and in kids, where experience of heparin anticoagulation is available. The replacement of warfarin in cancer patients having thrombosis and in other patients needing longstanding therapy LMWH has been evaluated as efficacious and safe as the first line choice and also secondary if warfarin or other anticoagulants have failed, i.e. a new thrombosis or an extension

of thrombosis has occurred (Carrier *et al.*, 2014). This can take place in cancer, typically also in association with antiphospholipid antibody syndrome, which may further complicate the thrombogenicity of cancer itself (Ortel *et al.*, 2015).

The data on new oral anticoagulants in cancer patients are scanty and the drugs may interact with oncological therapies causing harmful side effects and impairment of pharmacokinetics (Carrier *et al.*, 2014). The efficacy and safety of heparins in cancer patients also for long-term therapy have proven beneficial, especially in comparison with vitamin K antagonists.

As heparin is of animal origin and the attempt to produce it synthetically or by molecular means have been difficult, some minimal protein contamination is possible, which may cause rash or other immunological reactions, rarely even up to anaphylaxis. The only clinically really relevant specific side effect of heparins, especially the larger molecular weight species, is thrombocytopenia, which is triggered by immunological mechanisms. The epitope is mainly platelet-derived PF4, which avidly binds to heparin and neutralizes its effect. PF4 is a leukokine, which is released from activated platelets. Heparin PF4 creates immunoglobulin G4 (IgG4) antibodies, which activate platelets and endothelial cells and paradoxically generate thrombin. The key coagulation enzyme, thrombin itself or its formation is the target for the inhibition exerted by any anticoagulant, thereby the paradox is obvious. PF4 has also shown to be a negative regulator of megacaryopoiesis (Lambert *et al.*, 2009).

Bleeding complications are the common side effects of all anticoagulants. One needs to take into account the common precautions to avoid bleeding tendency. These include the diagnosis of anemia and correction of its cause. Iron deficiency is common and should be acutely managed, for instance by intravenous ferrous supplementation. Also, folic acid and vitamin B may be deficient spontaneously or triggered by pharmacological cancer therapies, and need to be controlled. Vitamin K-deficiency may develop after long standing parenteral nutrition and antibiotics. One needs to exclude other hematological diseases when differential diagnosis of thrombocytopenia is discussed (Table 3.1).

3.3.1 Characteristics of heparin-induced thrombocytopenia

The pathogenesis of HIT engages with the antibodies recognizing PF4/heparin complexes and activating platelets and endothelial cells and turn them procoagulant (Figure 3.1). Upon thrombin generation and with the Fc-receptor activation platelet membrane scrambles and the platelet surface remains negatively charged. This leads to assembly of coagulation factors on the platelet membrane to generate further thrombin. Platelets and monocytes are also known to microvesiculate further perpetuating the thrombotic syndrome (Kasthuri *et al.*, 2012).

HIT should be suspected early in case of sudden thrombocytopenia develops, usually within 5-10 days of the initiation of heparin administration. This may be challenging, as often times hematological malignancies and other cancer types and their treatment may induce thrombocytopenia (Ortel *et al.*, 2015). Usually the exclusion of other causes for thrombocytopenia

Table 3.1. Differential diagnosis of heparin-induced thrombocytopenia.[1]

Mechanisms		
Antibody-mediated mechanisms		**Infections, GVHD**
	TTP	antibodies against ADAMTS13
	Sulfa antibiotics	antibodies against the drug, this list is limited
	Carbamazepine	
	Kinin, Kinidin	
	Vancomycin	
Viral infections		
	Hantaan virus	pulmonary and renal disease, complement activation, consumption, endothelial binding
	HIV	
	Hepatitis C	other liver diseases, Ashwell receptor binding
	Cytomegalo virus	
Bone marrow failure		hematological and oncological therapies
	Drug-induced	
DIC		sepsis or other defined risk factor, prothrombin consumption, D-dimer elevation
cAPS		severe phospholipid antibody formation and multi-organ failure
aHUS		factor H deficiency and complement-induced activity
PNH		CD55 and 59 deficiency to regulate the complement system
Elevated PF4	Strong platelet activation	suppression of megakaryopoiesis

[1] ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, enzyme modifying von Willebrand factor multimer size; aHUS: atypical hemolytic uremic syndrome; cAPS: catastrophic antiphospholipid antibody syndrome; CD55/59: cluster differentiation antigens 55/59; DIC: disseminated intravascular coagulation; GVHD: graft-versus-host disease; PNH: paroxysmal nocturnal hemoglobinuria; TTP: thrombotic thrombocytopenic purpura.

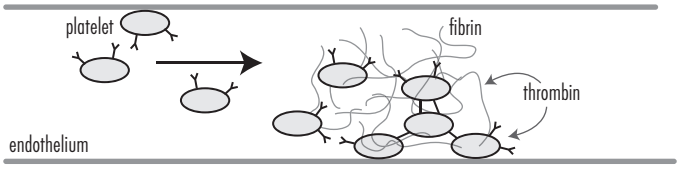

Figure 3.1. Platelets and endothelium are activated by the heparin-induced thrombocytopenia antibodies (Y). Upon activation they become procoagulant, may shed vesicles and lead to vessel occlusion due to uncontrolled thrombin generation and fibrin formation. Fibrin increasingly captures platelets and deepens thrombocytopenia.

strengthens the HIT diagnosis, but this may not apply among the cancer patient population. Thereby the timing of thrombocytopenia, in relation to the timing of myeloablative or megakaryocyte dampening therapies, appearance of new thrombosis, which may also turn out to be asymptomatic and can be picked up as incidentaliomas, are of major importance.

The incidence of HIT depends on the clinical situation. Cardiac surgery patients have an incidence of 1-5%, whereas medical and obstetric patients have 5-10-fold less incidence (Linkins *et al.*, 2012). Females carry twice the risk in comparison with males (Warkentin *et al.*, 2006)

The extent of thrombocytopenia can be modest, only 30% or more, but seldom very low platelet counts are detected (such as 20×10^9/l). Also, extracorporeal perfusions, such as dialysis or plasma exchange or cardiopulmonary bypass surgery or other major surgery may be an additional trigger of the HIT. This could also be major orthopedic surgery, such as is needed during pathological fractures or other exposure of tissue to blood. On the other hand, sometimes only a minor exposure to heparin in the form of catheter flushing or locks may be enough to trigger HIT.

In HIT the bleeding tendency is seldom a problem and platelet transfusions should be avoided. In our study of cardiac patients who underwent recent cardiopulmonary bypass, if platelets were transfused during the procedure the risk of HIT was enhanced (Kuitunen *et al.*, 2007). Once the diagnosis is made foreign platelets may not be a good idea for recovery as immunological reactions may become enhanced. Overall, platelet transfusions should be reserved only for bleeding complications and urgent major surgery, which carries a high bleeding risk (Linkins *et al.*, 2012).

3.4 Diagnosis of heparin-induced thrombocytopenia

The rule of four 't's (4Ts) has been recently established to suspect and confirm the diagnosis of HIT (Table 3.2) (Crowther *et al.*, 2010). The main problem in HIT is its delayed diagnostics, which worsens the outcome. The mortality in otherwise co-morbid patients is as high as 40%, but with an early diagnosis, avoidance of heparin and replacing anticoagulation with another drug, either a direct thrombin inhibitor argatroban or bivalirudin in case of cardiac thrombosis or interventions, or danaparoid, which is a heparinoid carrying other than antithrombin-activating potential as well.

Thrombocytopenia is the most obvious sign of HIT when associated with the use of heparin during the past 5-10 days, which time it takes to generate the antibodies against the heparin-PF4 complex. If the person has previously been exposed to heparin there is the possibility that the antibodies are present and the thrombocytopenia may occur quite instantly. Unusually, though, this is the sign of heparin-associated thrombocytopenia (HAT), which is transient and is not causing the HIT and HITT, the latter being thrombosis-associated thrombocytopenia.

Although not in the diagnostic criteria, as the sign of thrombin generation D-dimer is also elevated (Kuitunen *et al.*, 2007), albeit the thrombolytic capacity may be poor due to the strong platelet

Table 3.2. 4Ts score to obtain a clinical diagnosis of heparin-induced thrombocytopenia (a simplified version from Linkins *et al.*, 2014).[1]

	Score 2	Score 1
Thrombocytopenia	>30% from the original	lower nadir >20×10^9/l platelet count, nadir >20×10^9/l
Timing of thrombocytopenia and/or thrombosis, platelet fall	usually 5-10 days from onset of heparin 1 day from the onset of heparin if prior exposure	>10 days
Thrombosis	confirmed, new	recurrent erythema at injection site
Other mechanisms of thrombocytopenia unlikely or excluded	no alternative mechanisms	another mechanism is possible

[1] Less than 3 points: heparin-induced thrombocytopenia (HIT) is unlikely; 3-5 points: intermediate HIT risk; 6-8 points: HIT is definite. Up to 50% may have the 4Ts points between 3 and 5 and yet HIT may not be the diagnosis (Linkins *et al.*, 2014, Smock *et al.*, 2014).

activation and the release of plasminogen activator inhibitor-1 (PAI-1) hampering fibrinolysis. In deviation from disseminated intravascular coagulation, DIC, PT is usually normal, thus there is not a sign of consumption (Table 3.1). Another differential diagnostics is related to thrombotic thrombocytopenic purpura, where the symptoms are mainly related to microvascular thrombosis in cerebrovascular and renal area. Overt thrombosis is absent whereas the microvascular thrombosis consume platelets and causes an organ failure. Atypical hemolytic uremic syndrome is caused by complement activation, which may be linked to many of the conditions listed, such as DIC, TTP and also to some extent in HIT. On the other hand, heparin under normal conditions is also able to downplay the complement activation, and if pathological antibody formation ensues, overwhelming thrombin is generated, able to activate the complement protein C5 to C5a.

The most frequent thrombosis in HIT is venous thromboembolism (17-55%) (Linkins *et al.*, 2012): deep vein thrombosis of the extremities, catheter-related thrombosis or pulmonary embolism. Also, rare forms of thrombosis may occur, such as sinus thrombosis, mesenterial, portal, renal, adrenal, ovarian, subclavian and jugular vein thrombosis, and typically the thrombosis has multiple locations. Also, simultaneously or separately arterial thrombosis may occur (3-10%) (Linkins *et al.*, 2012), i.e. myocardial infarction, stroke, or sudden worsening of peripheral arterial occlusive disease or mere arterial thrombosis in lower extremities, with a threat of amputation. In addition to HIT, other situations where both venous and arterial thrombosis occur simultaneously may appear: the thrombotic burden may be increased by cancer, catastrophic antiphospholipid antibody syndrome, and rare and severe deficit of natural anticoagulants, such as antithrombin,

protein C and S deficiency, occurring in severe infections and severe hyperhomocysteinemia. HIT-associated limb necrosis is likely due to impairment of activated protein C system to regulate the distal perfusion both in arteries and veins.

3.4.1 Laboratory diagnosis of heparin-induced thrombocytopenia

In the clinical suspicion the diagnosis of HIT is based on the laboratory demonstration of the antibody formation in an ELISA assay. However, only a small proportion of patients who form HIT antibodies (seroconversion) will develop thrombocytopenia, and again an even smaller proportion will develop HIT-associated thrombosis. A rapid screening of HIT by latex-type of test in association with the proper clinical diagnostics of HIT is useful to exclude HIT or suggest further testing in the form of ELISA. The latex test (the ID-PaGIA Heparin/PF4 antibody test®; DiaMed, Cressier, Switzerland) is reliable as an exclusion test, with the sensitivity is 100%, however, over-diagnosis, as with ELISAs may occur (Lassila *et al.*, 2011, Linkins *et al.*, 2012). At on-call hours the exclusion opportunity is important, and the clinical picture should dominate the decision making. Recently the performance of ELISA assays among different North American laboratories indicated a good precision in clearly negative and positive samples, however, the challenge in the intermediate HIT remains (Smock *et al.*, 2014).

The cumbersome platelet aggregation test where the serum of the HIT patient will induce spontaneous aggregation of donor platelets, and the assay is modified by the exogenous heparin concentration, is considered the golden standard of laboratory diagnosis in HIT. However, the results may take several hrs or even days to obtain. The precision of serotonin release and platelet aggregation assay is far from optimal according to an exercise conducted recently by external quality control of diagnostic assays and tests (ECAT) (unpublished data).

With the rapid HIT test result being positive, and the clinical suspicion high, heparin should be stopped and an alternative anticoagulant should be started. The dosing and the choice of the alternative depend on the presence or absence of thrombosis, i.e. whether the anticoagulant is to be given at a thromboprophylatic or a full treatment dose. Thereby the extent of the thrombotic burden is to be carefully considered.

3.5 Management of heparin-induced thrombocytopenia

It is critical to understand whether there is active thrombosis or not. During HIT without thrombosis thromboprophylaxis is always recommended in hospitalized patients at least for 7-10 days or until the platelet count has normalized. Whether there is thrombosis or not will influence the dose and duration of anticoagulation. The first management issue is to select another non-heparin anticoagulant (Table 3.3). The selection is based on the patient characteristics.

When the diagnosis is clear all heparin exposures should be avoided, such as what is used in the catheter locks or to flush the catheter. Direct thrombin inhibitor can be also used in the

Table 3.3. Non-heparin anticoagulants for managing heparin-induced thrombocytopenia.[1]

	Considerations	Monitoring
Warfarin	PO, when platelet count has exceeded 150×10^9/l	INR
Argatroban (Novastan®)	IV, preferred in renal insuf ciency	
	not recommended in liver failure	APTT or diluted thrombin time assay with a drug standard
Bivalirudin (Angiomax®, AnigoxR)	IV, in cardiac patients, fast on – fast off action	APTT or diluted thrombin time assay with a drug standard
	in cardiac surgery UFH can be used with antiplatelet therapy for the period of the operation	
Danaparoid	heparinoid, antithrombin and heparin cofactor II -based (Orgaran®) action,	
	IV or SC a good alternative	anti-FXa activity
	reduced dosing in renal insuf ciency	assay with a drug standard
Fondaparinux	pentasaccharide	anti-FXa activity
	(Arixtra®) a potent FXa inhibitor via antithrombin	assay with a drug standard

[1] APTT: activated partial thromboplastin time; FXa: coagulation factor Xa; INR: international normalized ratio of prothrombin time assessment; IV: intravenous administration; PO: *per os*, oral administration; SC: subcutaneous injection; UFH: unfractionated heparin.

catheter locks, but usually the systemic infusion of the HIT-specific anticoagulant is enough to anticoagulate the patients efficiently and safely. It is also possible to use regional citrate to anticoagulate renal replacement therapy and catheter locks.

3.5.1 Thromboprophylaxis

Asymptomatic venous thromboembolism should be excluded with ultrasonography prior to switching the anticoagulant. For thromboprophylaxis in general and in pregnant patients subcutaneous danaparoid is recommended (Linkins *et al.*, 2012). Fondaparinux is a subcutaneously administered potent antithrombin-dependent FXa inhibitor, which may offer good and potent alternative thromboprophylaxis. There are incidental reports on the cross-reactivity of both danaparoid and fondaparinux with the HIT-antibodies, but clinically this is not usually meaningful. Platelet count monitoring on daily basis when treating thrombosis is necessary, while during thromboprophylaxis platelet count monitoring every 2-3 days is enough, until HIT reverts. Thromboprophylaxis should continue at least for 30 days and after delivery at least six weeks, and even longer periods up to 3 months, if the clinical situation has been complicated.

When taking care of patients having HIT, the typical watchful strategy to uncover thrombosis and bleeding risks is mandatory. Thus, blood cell counts not only to screen the platelet count outcome but also to ensure that red cells and leucocytes would be normal. Anemia impairs oxygen delivery and tissue perfusion impairing organ function, and anemia may also lead to bleeding risk due to impaired platelet adhesion to vascular injury sites. This is not the case usually in HIT, as von Willebrand factor levels may be elevated and enhanced thrombin generation ensures hemostasis. Also, the screening tests of coagulation, such as PT, APTT, D-dimer and antithrombin are recommended to be followed during the active HIT and its recovery. Usually the values normalize when the recovery is occurring. The thrombin inhibiting anticoagulant, argatroban or bivalirudin prolong the PT, but they can also impair vitamin K metabolism and/or liver function.

3.5.2 Treatment of thrombosis in heparin-induced thrombocytopenia

A rapid switch to a non-heparin anticoagulant with an effective dose is mandatory to achieve recovery from HIT, the mortality of which is still significant at least in association with comorbidities and after cardiac surgery. The choice of the heparin replacing anticoagulant depends on the clinical situation and the renal and liver function of the patient (Table 3.3). The duration of active intravenous infusion with a thrombin inhibitor or with danaparoid is usually one to two weeks. After that a switch to either subcutaneous danaparoid or warfarin or fondaparinux is possible and careful follow-up of the patient is important for a few weeks in order to confirm the appropriate recovery.

The monitoring of the systemic anticoagulant is needed on regular basis, on the first days 3-6 times daily, later 2-3 times, if the patient and the infusion are stable. Typically the platelet count starts to rise gradually, daily at least by 10%, which is the production rate of new platelets by the bone marrow. If the bone marrow is insufficient, obviously it will be challenging to evaluate the condition properly.

If bleeding ensues, which is untypical for HIT, or if there is a need for an invasive procedure, platelet transfusions may be administered. Overall, otherwise platelet transfusions are not recommended during HIT, as this may exacerbate the immune reactions (Kuitunen *et al.*, 2007, Linkins *et al.*, 2012).

If the patient is on warfarin, while HIT is diagnosed, it is preferable to convert the regimen to a HIT-compatible anticoagulant according to the clinical situation. Then vitamin K should be given to recuperate the warfarin action, usually 1-3 mg intravenously or 3 mg perorally is a suitable dose. The response to vitamin K should be checked within 12-24 hrs, and then if the liver is producing the coagulation factors normally and there are no signs of consumption coagulopathy or liver insufficiency, the PT should be normalized.

INR is designed for warfarin monitoring only, and it is not reliable to mirror liver function. In addition, Owren and Quick buffers in the PT and INR are used in the different parts of the world

and they differ in that Quick is sensitive to FV deficiency, which indicates liver failure more strongly than is the case with the Owren buffer, which is insensitive to FV levels.

When the time to resume warfarin is actually, usually quite urgently in case of mechanical heart valve, HIT-specific anticoagulant can be switched to warfarin once the platelet count has normalized, i.e. reached the level above 150×10^9/l. Under normal circumstances this takes 7-10 days, if the thrombosis reacts to the thrombin inhibition achieved with a non-heparin anticoagulant and the antibody formation ceases. This time refers to the circulating lifetime of platelets and usually during one week the immune reaction starts to taper down.

If the syndrome is severe, sometimes a few weeks are needed to stabilize the situation, and occasionally other anti-inflammatory therapies, such as corticosteroids and or immunoglobulins are needed. When switching the HIT-specific anticoagulant to warfarin, an overlap of at least 5 days is needed to cover the period when protein C and S, the vitamin K dependent naturally occurring anticoagulants are lowered (usually days 1-3) prior to the main procoagulant factors, i.e. prothrombin and FX are affected by warfarin (usually by 5 days).

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4. Management and outcomes in severe anemia

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Abstract

Anemia is a worldwide public health concern affecting people of all ages and socio-economic status. Iron-deficiency anemia is one of the most important contributing factors to the global burden of disease affecting about two billion people and the cause of approximately one million deaths annually. Anemia in hospitalized patients is common and often multi-factorial. It is associated with increased morbidity, mortality and utilization of health care resources. Management of anemia includes treatment of its cause, administration of alternatives to blood transfusion, allogeneic red blood cells transfusion, optimizing nutrition, mineral supplementation and treatment of anemia-related complications. Jehovah's Witnesses (JWs) with acute blood loss in the community is a medical emergency. They are directed to a high acuity area of the emergency department for immediate assessment by a multidisciplinary team and treatment. Hemodynamically unstable bleeding patients should undergo life-saving surgical hemostasis, whereas hemodynamically stable patients emergency hemostasis. High risk for re-bleeding, hemodynamically unstable and high mortality risk patients should be admitted to intensive care and high dependency unit for monitoring and treatment. High dosage regime of erythropoietin and iron supplementation should be used in severely anemic JW patients for erythropoiesis stimulation. Infective complications should be promptly diagnosed and treated. Doctor-JWs patient interaction is a balance between the deliberate and interpretive models of doctor-patient relationship. During meetings, clinicians should disclose mortality risks to the patient and his/her family and confirm whether the patient continues object to life-saving blood transfusion. Blood transfusion is the primary treatment for ongoing bleeding with hemodynamic compromise, hemorrhagic shock and severe anemia. Blood transfusion is associated with reduced rate of cardiac, neurological and infective complications, and utilization of health care resources. Anemia of critical illness is managed with minimization of blood loss, optimizing nutrition, mineral supplementation, a restrictive blood transfusion strategy, and other supportive measures as required.

Keywords: blood loss, hemorrhagic shock, Jehovah's Witnesses, blood transfusion alternatives, doctor-patient relationship

Key facts

- Anemia, particularly iron-deficiency anemia, is one of major causes of global disease burden affecting an estimated two billion people of all ages, but pregnant and postpartum women and young children are the most vulnerable. Iron-deficiency anemia is estimated to be the cause of approximately one million deaths per year worldwide.
- In hospitalized patients anemia is common and often multi-factorial.
- Severely anemic patients treated with alternatives to blood transfusion, including erythropoietin, iron, vitamin B12, folate supplementation and supportive measures have 20% mortality and significant cardiac, neurological and infective morbidity.
- Blood transfusion for treatment of severe anemia is life-saving, the number needed to treat to prevent one death is six, and cost-effective. It has the incremental cost-effectiveness ratio of 2011 US$ 22,515 for each death prevented.
- Anemia of critical illness is multi-factorial. It is managed with prevention of blood loss, stimulation of erythropoiesis, optimizing nutrition and supportive measures.

Summary points

- Anemia is a common problem representing a large healthcare burden, morbidity and mortality risk worldwide.
- In clinical settings if severe anemia is not treated with blood transfusion is associated with up to 20% mortality, increased morbidity and utilization of health care resources.
- Management of severe anemia includes treatment of its causes, blood transfusion, administration of alternatives to blood transfusion, allogeneic red blood cell transfusion, optimizing nutrition, mineral supplementation and treatment of complications of anemia, such as infection, etc.
- Optimal triggers for blood transfusion is not yet established, but blood transfusion decisions should be individualized and consider not only hemoglobin concentration, but also patient's co-morbidities and tissue oxygenation.
- Severely anemic Jehovah's Witness patients present a medical emergency and should be assessed and treated by a multidisciplinary team.
- A new management algorithm for Jehovah's Witnesses with acute blood loss has been proposed that includes different pathways for hemodynamically stable and hemodynamically unstable patients.

Abbreviations

AMRS	Anemia mortality risk score
ARBC	Allogeneic red blood cell
CaO_2	Oxygen content of blood
CBC	Complete blood count
CI	Cardiac index
DAA	Direct-acting oral anticoagulants
DAT	Dual antiplatelet therapy
DO_2	Total oxygen delivery
EBL	Estimated blood loss
EBV	Estimated blood volume
ED	Emergency department
EPO	Erythropoietin
FFP	Fresh frozen plasma
Hb	Hemoglobin
HDU	High dependency unit
ICU	Intensive care unit
IL-6	Interleukin 6
JW	Jehovah's Witness
PaO_2	Partial pressure of oxygen
PCC	Prothrombin complex concentrate
RPI	Reticulocyte production index
SaO_2	Hemoglobin oxygen saturation
WHO	World Health Organization

4.1 Introduction

The WHO defines anemia as 'a condition in which the number of red blood cells (and consequently their oxygen carrying capacity) is insufficient to meet the body's physiological needs' (WHO, 2011). Anemia may be classified as mild, moderate, or severe, based on Hb concentration. As the physiological needs of individuals vary with age, gender, pregnancy, altitude of residence, and smoking, Hb concentrations used to define anemia vary accordingly. The WHO definitions of anemia are presented in Table 4.1.

Anemia is associated with worsened health outcomes, including increased morbidity and mortality, increased hospitalization rates, and reduced quality of life (Carson *et al.*, 2002; Hayden *et al.*, 2012). In this chapter, the etiology, presentation, management and outcomes of patients with severe anemia are outlined and discussed.

Table 4.1. Hemoglobin levels to diagnose anemia at sea level (g/dl) (modified after WHO, 2011).

Population	Mild anemia	Moderate anemia	Severe anemia
6-59 months age	10.0-10.9	7.0-9.9	<7.0
5-11 years age	11.0-11.4	8.0-10.9	<8.0
12-14 years age	11.0-11.9	8.0-10.9	<8.0
Non-pregnant women (15 years age and older)	11.0-11.9	8.0-10.9	<8.0
Pregnant women	10.0-10.9	7.0-9.9	<7.0
Men	11.0-12.9	8.0-10.9	<8.0

4.2 Etiology

Anemia occurs due to decreased production of red blood cells, increased destruction of red blood cells, or loss of red blood cells. The most common cause of anemia worldwide is iron deficiency. Other common causes of anemia include other nutritional and mineral deficiencies, chronic illnesses (including cancers, kidney disease, infections and hemolysis), acquired or inherited disorders of blood production and regulation (such as drug therapies, bone marrow disorders, hypersplenism and pregnancy), and blood loss (either acute or chronic).

4.2.1 Causes of acute severe anemia

Blood loss

Blood loss is a common cause of anemia in hospitalized patients, particularly patients presenting to EDs, undergoing surgery, or critically ill patients. Blood loss may be rapid, such as associated with major trauma, surgery, or acute gastrointestinal or gynecological bleeding, ruptured aneurysms, or spontaneous bleeding following anticoagulant therapy, or may be slow such as associated with slower gastrointestinal losses such as from tumor or arteriovenous malformations, menorrhagia, or repeated blood sampling.

Importantly, acute blood loss will initially not alter the measured Hb, as whilst the intravascular blood volume will fall, the concentration of Hb will initially be relatively constant. As physiologic compensatory mechanisms or fluid resuscitation occur to restore intravascular volume, the measured hematocrit and Hb will then decrease. The EBV for adults is 7% of total body weight (70 ml/kg), with a 70 kg person having an EBV of 5 l. Categories of hemorrhagic shock according to EBL (prior to resuscitation) have been described, and these correlate with clinical findings and presentation with percentage EBL. These are summarized in Table 4.2.

Table 4.2. Classification of hemorrhage (modified after Committee on Trauma, 1997; Gutierrez *et al.*, 2004).

Parameter	Class I	Class II	Class III	Class IV
Blood loss (ml)	<750	750-1000	1000-2,000	>2,000
Blood loss (%)	<15	15-30	30-40	>40
Heart rate (beats/min)	<100	100-120	120-140	>140
Blood pressure (mmHg)	normal	decreased	decreased	decreased
Respiratory rate (breaths/min)	<20	20-30	30-40	>40
Urine output (ml/hr)	>30	20-30	<20	negligible
Central nervous system symptoms	normal	anxious	confused	lethargic or obtunded

An estimate of the volume of blood loss (or the effect on hematocrit) in acute hemorrhage with fluid resuscitation is possible, using Equation 1 (Gutierrez *et al.*, 2004):

$$EBL = EBV \times (H_i/H_f) \quad (1)$$

Where H_i is the initial hematocrit value and H_f is the final hematocrit value.

This equation assumes no significant other plasma losses (such as third space leakage of fluid into the interstitium) and no significant urinary losses.

The underlying etiology of hemorrhagic shock is inadequate delivery of oxygen to tissues. DO_2 is dependent on CI and CaO_2. The CaO_2 is in turn dependent on Hb level and SaO_2, and the PaO_2. This is summarized by the Equation 2 and 3:

$$DO_2\ (ml/O_2/min/m^2) = CI\ (l/min/m^2) \times CaO_2\ (ml/O_2/l\ blood) \quad (2)$$

$$CaO_2\ (ml/O_2/l\ blood) = 13.4 \times [Hb\ (g/dl)] \times SaO_2 + 0.03\ PaO_2 \quad (3)$$

Inadequate DO_2 occurs in acute hemorrhage secondary to hypovolemia (due to reduced cardiac output and tissue perfusion), but also (in the presence of isovolumetric fluid resuscitation) with anemia (due to reduced oxygen carrying capacity of blood). In fact, it is the critical level of DO_2 that is important in terms of physiologic effects of acute blood loss or anemia, and this level is essentially constant, at 8-10 ml/min/kg regardless of etiology (hypovolemia, hypoxia, and anemia) of inadequate DO_2. Of note, the WHO definition of anemia reminds us that it is the DO_2 that is relevant and important. Studies have demonstrated that a critical DO_2 is reached at Hb concentration of between 4 to 5 g/dl (Cain and Chapler, 1988; Gutierrez *et al.*, 1990).

Dilution

Dilutional anemia may occur in the setting of fluid resuscitation following or during acute hemorrhage or trauma, or in the setting of iatrogenic over-administration of fluid in trauma, or the perioperative setting, or as a normal physiological event in pregnancy. Other less common causes of dilutional anemia include congestive cardiac failure, massive splenomegaly, and exercise-induced – these dilutional anemias are due to expansion of the plasma volume (relative to red blood cell mass) within the intravascular compartment (Hess *et al.*, 1976; Shaskey and Green, 2000).

Critical illness

Anemia in critical illness is multifactorial in etiology. Pre-existing illness, surgery, or trauma may cause anemia prior to admission to ICU. The exact prevalence of anemia on admission to ICUs is unknown, and likely varies with ICU population and casemix, but some studies have reported 60-70% of patients admitted to ICU have Hb concentration <12 g/dl, and up to 30% of patients with Hb concentration <9 g/dl (Corwin *et al.*, 2002; Vincent *et al.*, 2002; Walsh *et al.*, 2006).

During ICU admission ongoing fluid resuscitation and hemodilution, blood loss due to trauma or surgery, blood loss due to frequent sampling, chronic inflammation, infections, renal impairment and reduced EPO production, nutritional deficiencies or abnormalities, abnormal erythropoiesis, and use of medications may contribute. It is estimated that patients lose up to 40-100 ml of blood per day due to blood sampling in the ICU, most of which is discarded, whilst the normal bone marrow makes 15-20 ml of blood per day (Vincent *et al.*, 2002; Von Ahsen *et al.*, 1999). When erythropoiesis is abnormal, and other sources of loss are present, critically ill patients may quickly become anemic. One study reported that in the first three days of ICU admission in non-bleeding patients, Hb concentrations decrease by a mean of 0.5 g/dl per day (Nguyen *et al.*, 2003).

4.2.2 Causes of chronic severe anemia

Iron deficiency

Iron deficiency is the most common worldwide cause of anemia, and may cause severe anemia. Iron deficiency is estimated to affect more than two billion people worldwide, and as many as one billion people are estimated to have iron deficiency anemia (Camaschella, 2015; WHO, 2002). Inadequate dietary iron intake or absorption, or chronic blood loss leads to the reduction of iron stores within the body, a decrease in total iron levels, and impaired erythropoiesis leading to a production of hypochromic microcytic red blood cells.

Iron deficiency anemia differs from both iron-restricted erythropoiesis and functional iron deficiency, which are states of altered iron homeostasis that may also produce anemia.

Iron deficiency anemia is most commonly caused by inadequate dietary intake. Malnutrition, vegetarian or vegan diets, diets low in heme (animal source) iron, and diets with no iron fortification are associated with iron deficiency. Those with an increased physiologic demand for iron are particularly at risk, including children, adolescents, and menstruating or pregnant women. Other causes of iron deficiency include decreased gastrointestinal absorption of iron, chronic blood loss, drug related iron deficiency, genetic disorders of iron metabolism, and iron-restricted erythropoiesis due to chronic inflammation or disease.

Decreased absorption occurs due to gastrectomy or atrophic gastritis, duodenal bypass or inflammatory bowel diseases, and *Helicobacter pylori* infection. Absorption is also affected by vitamin C – with reduced absorption in vitamin C deficiency.

Causes of chronic blood loss include gastrointestinal tract bleeding, some infections (nematodes, etc.), genitourinary bleeding, and hemolysis. Drugs that may cause iron deficiency include glucocorticoids and proton-pump inhibitors.

Vitamin B12/folate deficiency

Vitamin B12 (cobalamin) and folate are both essential in normal bone marrow function and erythropoiesis. Vitamin B12 and/or folate deficiency cause megaloblastic anemia, with macrocytosis, presence of immature megaloblastic nucleated red blood cells, and a normal or raised mean corpuscular volume seen in peripheral blood, whilst a hypercellular and dysplastic pattern is seen in bone marrow samples.

Abnormal red blood cell production causes increased destruction and intramedullary hemolysis (Stabler, 2013). Importantly, vitamin B12 is involved in normal cellular processes throughout the body, and deficiency can also cause demyelination in the central and peripheral nervous system, and is associated with infertility and thrombosis (Stabler, 2013).

Vitamin B12 is synthesized by microorganisms, ingested in the diet, and absorbed in the gastrointestinal tract. Intrinsic factor (synthesized by gastric parietal cells) is essential to allow absorption.

The most common causes of vitamin B12 deficiency are autoimmune atrophic gastritis (resulting in a lack of intrinsic factor) and a vegan or vegetarian diet (as animal origin foods are higher in vitamin B12 than plant origin foods). Other causes include gastric or ileal resection or bypass, inflammatory bowel disease, chronic pancreatitis, drugs (such as gastric acid inhibitors, and metformin), and rare genetic disorders.

Other nutritional deficiencies

Also essential for normal erythropoiesis are trace amounts of vitamin C, riboflavin, copper and vitamin A. Significant deficiencies of these nutrients may cause anemia.

Infection, inflammation and anemia of chronic disease

The etiology of anemia in infection, inflammation and chronic disease is multifactorial. Infection triggers the local or systemic inflammatory response and the proliferation of cytokines, in particular IL-6. Increased IL-6 levels cause increased production and release from the liver of hepcidin. Hepcidin is a peptide protein with antimicrobial and iron regulation functions. In fact, hepcidin is the most important iron regulatory protein, responsible for the normal responses of iron storage cells (hepatocytes), iron absorption cells (enterocytes) and iron recycling cells (macrophages) to iron overload or depletion (Rossi, 2005). Hepcidin acts by binding to ferroportin, an iron export protein normally located on the cell surface of those cells involved in iron regulation. The binding of hepcidin to ferroportin causes ferroportin to be internalized from the cell membrane, and subsequently degraded. This causes decreased iron release into the circulation from enterocytes, macrophages and hepatocytes. This is a normal response to increased iron load in healthy individuals, but also an appropriate and beneficial response to infection as the reduced iron availability restricts invading organisms' ability to survive and reproduce. However, a prolonged state of infection or inflammation will lead to anemia. The anemia seen in chronic infections and inflammation is typically associated with a low transferrin saturation, decreased total iron binding capacity, low serum iron level, a normal or increased serum ferritin, and a normocytic anemia (Viana, 2011). The anemia of chronic disease is usually mild or moderate, rather than severe.

In addition, anemia of infection, inflammation and chronic disease is associated with a reduced production of EPO and impaired bone marrow response to EPO.

Also in systemic infections bone marrow hematopoiesis becomes altered causing an increased production of monocytes and granulocytes, and maturation arrest of lymphoid and erythroid lineages causing anemia (Glatman Zaretsky *et al.*, 2014).

Finally, in inflammatory states the circulating red blood cells may have increased destruction and shortened half-lives due to oxidative damage and increased sequestration, further contributing to the development of anemia.

Infection may also cause anemia by direct mechanisms including direct invasion of red blood cells and production of hemolysins. Hemolysins are proteins produced by many bacteria and fungi, capable of destruction of red blood cell walls, allowing invading organisms' access to iron, and in some cases allowing organisms to evade specific host responses to infection. Infections commonly causing hemolysis include cytomegalovirus, Epstein Barr Virus, typhoid, *Escherichia coli*, *Mycoplasma pneumoniae*, and *Streptococcus*.

Antimicrobial therapy may also cause anemia by several mechanisms. Zidovudine (azidothymidine) inhibits erythropoiesis, whilst penicillins, sulfur drugs, and antimalarial therapies can all cause hemolysis.

Chronic kidney disease causes anemia by several mechanisms. Reduced production of EPO produced by the kidneys, both de novo and in response to anemia, an impaired bone marrow response to EPO and an iron deficiency state leads to inadequate red blood cell production. Decreased life-span of red blood cells and blood loss due to frequent blood sampling, consumption of red blood cells in a dialysis circuit and bleeding secondary to platelets dysfunction are important contributing factors in anemia of chronic kidney disease.

Hemolytic anemias

Both inherited and acquired conditions may cause hemolysis. Hereditary hemolytic anemias are due to red blood cell membrane defects (hereditary elliptocytosis and hereditory spherocytosis), due to abnormal metabolism of red blood cells (pyruvate kinase deficiency and glucose-6-phosphate dehydrogenase deficiency) and secondary to defects in Hb production (sickle-cell disease, thalassemia).

Acquired hemolytic anemia may be immune-mediated (cold agglutinin disease and autoimmune hemolytic anemia), complement-mediated (paroxysmal nocturnal hemoglobinuria), caused by hypersplenism and other heterogenous causes.

Drugs and toxins such as gold, sulfonamides, penicillins and antimalarials may also cause hemolysis, as may radiation exposure or blood transfusion reactions. Viral infections such as Human Immunodeficiency Virus and hepatitis can trigger hemolysis, as may mechanical conditions such as valvular heart disease, prosthetic heart valves, or marathon running.

Bone marrow and blood disorders – inherited and acquired

Many disorders of the blood and bone marrow can cause severe anemia. Myelodysplastic syndromes, myeloproliferative disorders, leukemias, lymphoma, and aplastic anemia all cause anemia and sometimes pancytopenia. Infiltration of bone marrow by other primary cancers may impair hematopoiesis directly. Finally, hypersplenism causes increased sequestration and destruction of red blood cells resulting in anemia.

4.3 Presentation

The presentation of patients with severe anemia varies according to onset and duration, as well as patient co-morbidities, and therapies.

4.3.1 Acute severe anemia

Acute blood loss is variably tolerated according to volume of blood loss, subsequent fluid replacement and resuscitation, resultant hematocrit, and an individual's baseline Hb concentration and co-morbidities. Acute blood loss >15% of EBV is associated with the first

signs of hemodynamic and respiratory compromise, and losses >30% are associated with signs of inadequate end-organ perfusion (Table 4.2).

Pathophysiology of acute blood loss and hemorrhagic shock

The effects of acute blood loss vary according to percentage of circulating volume lost, and compensatory mechanisms' effectiveness, along with subsequent therapeutic interventions. A loss of 10% of the total euvolaemic blood volume, approximately equivalent to a standard blood donation, has little effect on cardiac output and arterial pressure and usually produces no features of clinical concern. A loss of 20 to 30% of circulating blood volume initially reduces cardiac output, then arterial pressure, and causes clinical shock. A loss of 30 to 40% of blood volume causes severe hypotension, and progresses to end organ compromise manifested by confusion, agitation or obtundation, cardiovascular and respiratory instability, and anuria (Levick, 2003). Acute blood loss greater than 45% of the total blood volume is usually lethal (Hall, 2011).

In acute hemorrhage the reduction in blood volume causes a decrease in end-diastolic volume and a reduction in stroke volume and pulse pressure. The mean arterial blood pressure is initially maintained; called compensated shock. The activity of arterial baroreceptors and cardiopulmonary stretch receptors decreases, whilst arterial chemoreceptors maybe activated by metabolic and respiratory acidosis due to reduced perfusion. Arterial chemoreceptor activation causes an increase in sympathetic vasomotor activity (via the nucleus tractus solitarius) and a raised concentration of vasoactive hormones, including epinephrine, norepinephrine, vasopressin, and angiotensin II. The physiological responses are tachycardia and increased myocardial contractility, along with vasoconstriction in the cutaneous, skeletal muscle, mesenteric and renal vascular beds. These responses partly compensate for the reduced cardiac output and arterial blood pressure. Vasoconstriction, however, may reduce tissue and organ perfusion and cause oliguria, lactic acidosis, or other signs of inadequate organ perfusion. A reduction in venous pressure causes sympathetic-mediated arteriolar constriction, and increases the precapillary to postcapillary resistance ratio, reducing capillary blood pressure. As a result, the plasma colloid osmotic pressure predominates and causes a transient absorption of interstitial fluid into the intravascular compartment, sometimes called the 'internal transfusion' or 'autotransfusion'. In addition, epinephrine triggers glycogenolysis in the liver, with release of glucose into the circulation. Increased glucose concentrations cause the plasma and interstitial fluid osmolarity to increase by up to 20 mOsm, drawing intracellular fluid into the interstitial compartment.

These initial mechanisms attempt to maintain adequate perfusion of vital organs. Subsequently, further mechanisms attempt to restore intravascular volume and red blood cell mass. Vasopressin and aldosterone stimulate salt and water reabsorption, angiotensin II activates the thirst mechanism, and an increased water intake. EPO is secreted by the kidney and stimulates red blood cell production by the bone marrow, which may restore normal Hb concentrations over several weeks.

Deleterious effects of these compensatory mechanisms may also occur. Peripheral vasoconstriction impedes blood flow and oxygen delivery to the visceral organs and tissues, stimulating anaerobic metabolism of glucose with subsequent lactate production and release into the circulation. Reduced lactate clearance may occur due to reduced hepatic perfusion, leading to a worsening metabolic acidosis and further depressing myocardial contractility and tissue perfusion. Decompensated shock develops when compensatory mechanisms are inadequate (Figure 4.1). Vasodilatation occurs due to the release of endogenous opioids, beta-endorphins and enkephalins, and a reduction in sympathetic outflow. Reduced coronary perfusion and worsening metabolic acidosis further impair myocardial contractility. In patients with coronary artery disease, the reduction in coronary blood flow may even cause myocardial ischemia or infarction. Decreased tissue perfusion leads to microcirculatory damage, cellular aggregation and microcirculatory obstruction. If hypoperfusion is prolonged, multiple organ failure and death may occur.

4.3.2 Chronic severe anemia

Patients with chronic severe anemia often present with non-specific symptoms. Fatigue, malaise, weakness, headaches or poor concentration, decreased exercise tolerance, exacerbation of angina

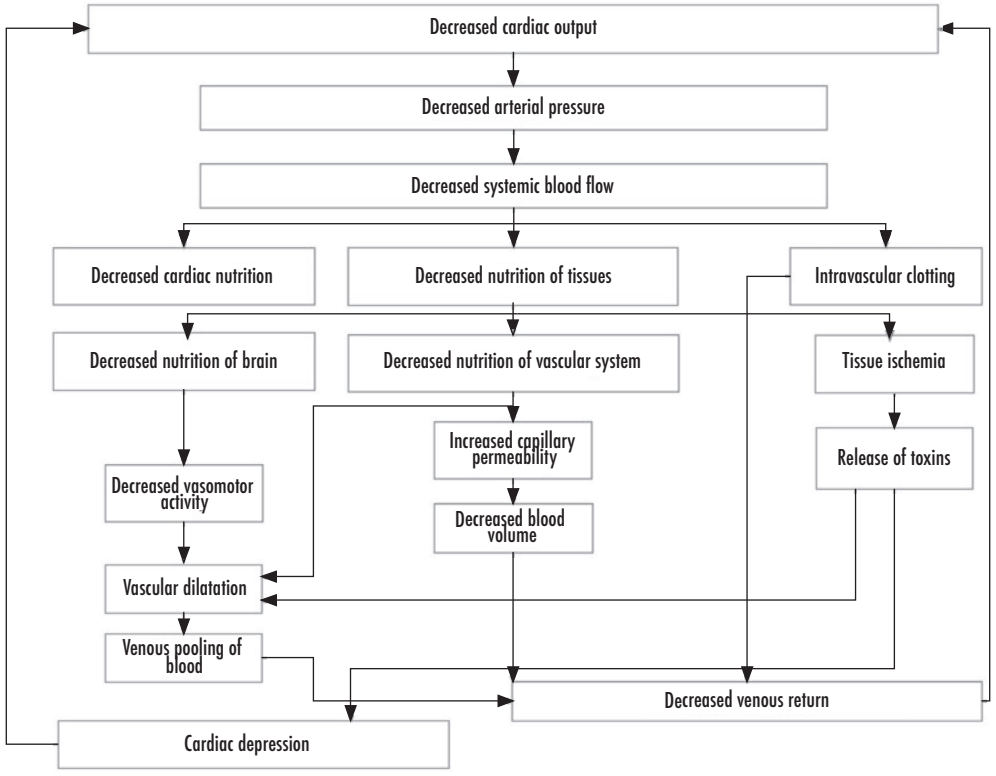

Figure 4.1. Pathophysiological consequences of hemorrhagic shock (adapted from Hall, 2011).

symptoms or congestive heart failure, arrhythmias, syncope or pre-syncope episodes may occur, however chronic anemia of gradual onset and worsening severity may be well tolerated, even with very low Hb concentrations (as low as 5.0 g/dl), due to compensatory physiological mechanisms and cellular metabolic changes in the presence of hypoxia.

4.4 Management

The management of severe anemia includes anemia prevention, treatment of its causes, treatment with alternatives to blood transfusion, blood transfusion aiming for restoration of tissue oxygen delivery, supportive measures, including treatment of anemia-related complications, and an effective doctor-patient relationship. Global anemia risk prevention measures are beyond the scope of this chapter and will not be discussed here.

4.4.1 Severe anemia prevention in hospitalized patients

Patients presenting for major elective surgery should have optimization of Hb preoperatively, with identification and treatment of cause of anemia. Risk factors for excess blood loss should be identified and addressed preoperatively also, in particular the use of antiplatelet or anticoagulant therapy. Patients undergoing major elective surgery who receive preoperative antiplatelet therapy may have increased risk for perioperative bleeding (Hastings *et al.*, 2015). Recommendations for discontinuation of antiplatelet therapy before major surgery should be followed (Douketis *et al.*, 2008; Eagle *et al.*, 2004; Rossini *et al.*, 2014). Mono and DAT and warfarin should be discontinued five days before major surgery (Eagle *et al.*, 2004; Fitchett *et al.*, 2009). Elective major surgery for patients with a recent coronary artery stenting, who receive DAT, should be postponed for at least six weeks. Duration of treatment interruption for elective surgical patients on DAA, dabigatran, rivaroxaban and apixaban, depends on risk of bleeding and creatinine clearance (Heidbuchel *et al.*, 2013). Bridging therapy with heparin as an alternative to warfarin is recommended for elective high-risk for thrombo-embolic complications surgical patients, but is not recommended for patients on DAT or DAA (Dunning *et al.*, 2008).

In JW patients, who refuse blood transfusion on religious grounds, presenting for surgery, minimally invasive surgical techniques such as transcatheter aortic valve implantation, off-pump coronary artery bypass, the use of electrosurgical, ultrasonic scalpels, Ligaclips, cell saver technology, local hemostatic and sealant agents should be considered and utilized as surgical blood conservation strategies.

4.4.2. Etiological treatment of severe anemia

Anemia is confirmed with laboratory CBC. The blood film microscopy is often useful in differentiating etiology (e.g. hypochromic microcytic red blood cells vs. macrocytic red blood cells). Other investigations are directed by history and clinical presentation and may include

nutritional and infection screening, bone marrow sampling and analysis, to endoscopic examinations, and genetic testing.

Vitamin and mineral deficiencies are managed with replacement. Iron deficiency is usually managed with oral iron supplementation (100 to 200 mg elemental iron daily for adults), but may require intravenous iron, which has increased efficacy in certain patient groups (e.g. chronic kidney disease), and may be ineffective in disorders of iron metabolism. Transfusion of red blood cells is indicated when severe symptoms are present (e.g. angina, heart failure). Note that transfusion of red blood cells corrects not only anemia, but also iron deficiency, as one unit of red blood cells contains approximately 200 mg of iron (Camaschella, 2015). Vitamin B12 and folate supplementation is usually intramuscular or intravenous, but may be in the form of high dose oral therapy (Stabler, 2013). Hemolytic anemias and disorders of blood and bone marrow are managed with therapy specific to underlying cause, however it is important to note that simple transfusion of red blood cells in a hemolytic anemia may not be efficacious and can lead to further hemolysis. Acute blood loss is managed with hemostasis, intravascular fluid resuscitation including transfusion of ARBCs and blood products, and supportive care. Critically ill patients benefit from ongoing care in a HDU or ICU.

Management of acute blood loss with blood alternatives

JW patients present a unique insight into severe anemia management. Ongoing bleeding in JW patients is a medical emergency. Patients with ongoing bleeding, hemodynamically unstable patients, and patients with significant blood loss should be immediately triaged and admitted into a high acuity area, such as the resuscitation area of ED (Figure 4.2).

A multidisciplinary team approach is helpful, with input from emergency physicians, critical care physicians, surgeons, interventional gastroenterologists, gynecologists, hematologists, or other specialists as appropriate. Thus, diagnosis and definitive treatment may proceed with maximum efficiency. Important in the clinical history is recent use of anticoagulant or antiplatelet therapy, or other risk factors for coagulopathy (e.g. hepatic disease, excessive alcohol use, nutritional deficiency). Subsequent physical examination and primary and secondary surveys are focused on identification of the bleeding source and assessment of the pathophysiological effects of bleeding. Investigations should include CBC, electrolytes including calcium, urea, creatinine, liver function tests, coagulation studies, arterial blood gas and chest X-ray.

Early control of hemorrhage is the primary focus. At this time, it is important if possible, to discuss and clarify the patient's wishes in regard to ARBC transfusion, transfusion of platelets, FFP, cryoprecipitate, recombinant factor VIIa and other blood products.

Bleeding warfarinized patients and patients on DAA require an emergency protocol-based anticoagulant reversal (Baker *et al.*, 2004; New Zealand Government, 2011). Warfarin reversal can be achieved with intravenous administration of vitamin K, transfusion of FFP, or PCCs (Baker *et al.*, 2004). However some JW patients may object to transfusion of FFP and PCCs, because these

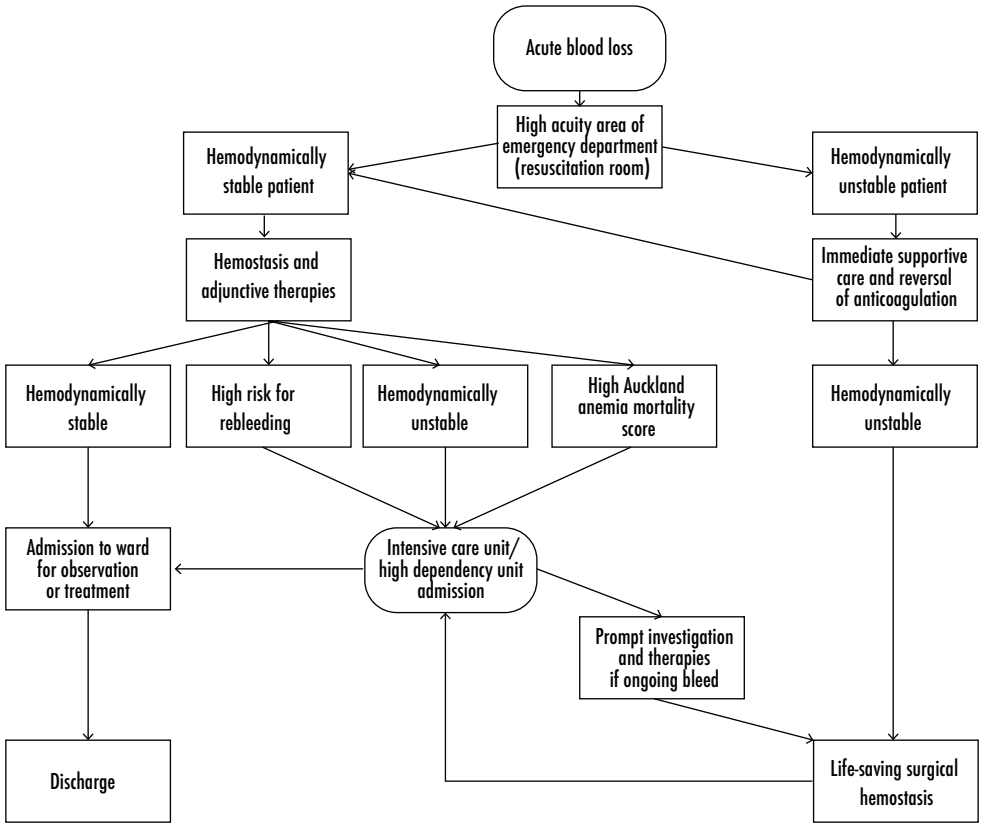

Figure 4.2. Management algorithm of acute hemorrhage in Jehovah's Witness patients.

products are manufactured using human plasma. PCCs (profilnine, prothromplex T) contain much higher concentrations of vitamin K-dependent clotting factors (II, VII, IX and X) than FFP, require less transfusion volume and have high efficacy, especially in emergency setting.

Hemodynamically unstable patients should proceed for a life-saving hemostasis. Hemodynamically stable patients with ongoing bleeding should proceed from ED to OR for local emergent hemostasis, such as upper endoscopy, colonoscopy or embolization of bleeding vessels, evacuation of retained products of conception, etc. Following the emergent hemostasis, patients with high risk for re-bleeding, hemodynamically unstable patients and patients with high Auckland AMRS are transferred to ICU/HDU for monitoring and treatment.

Auckland AMRS is calculated by calculating early mortality risk factors (Table 4.3) (Beliaev *et al.*, 2012b). Then mortality risk can be stratified according to Auckland AMRS groupings (Table 4.4).

Early mortality risk stratification can be helpful. For example, a 54 year old obese female patient with acute myocardial infarction and a recent pulseless electrical activity cardiac arrest

Table 4.3. Early mortality risk factors in severely anemic Jehovah's Witness patients and their risk weightings (modified from Beliaev *et al.*, 2012b).

Early risk factors of mortality	Weight
Age ≥45 years	1
Obesity (weight ≥90 kg)	1
Hypertension	1
Angina	1
Cardiac arrhythmia	1
Valvular heart disease	1
Previous myocardial infarction	1
Congestive heart failure	1
End-stage renal disease requiring hemodialysis	1
Acute hospital admission	1
Hemoglobin ≤8.0 g/dl	1

Table 4.4. Absolute and relative risks for mortality in severely anemic Jehovah's Witness patients according to their Auckland anemia mortality risk score (AMRS) (modified from Beliaev *et al.*, 2012b).

Auckland AMRS groups	Absolute risk of mortality (%)	Relative risk of mortality	*P*-value
0-3	4	1	–
4-5	32	7.2	0.028
6-7	50	11.3	0.0005
≥8	83	18.9	0.0005

requiring cardio-pulmonary resuscitation, hypertension, end-stage renal disease treated with hemodialysis, and chronic anemia with Hb of 7.5 g/dl (Auckland AMRS=7), needs an admission to ICU for ongoing monitoring and other supportive cares, an immediate cardiology review and intervention. However, a hemodynamically stable (pulse <100 beats per min and systolic blood pressure ≥100 mmHg) young healthy female patient who underwent complete evacuation of retained product of conception for a miscarriage with perioperative Hb of 7.0 g/dl and a low Auckland AMRS score may be safely discharged from hospital after fluid resuscitation and a short period (e.g. overnight) of observation.

Anemic JW patients in ICU/HDU and in the ward should be carefully observed and monitored for late anemia mortality risk factors (Table 4.5) (Beliaev *et al.*, 2013). The Hamilton AMRS can be calculated by combining weights of late risk factors and mortality risk stratified for different

Table 4.5. Late risk factors for mortality in severely anemic Jehovah's Witness patients with risk weightings (modified from Beliaev *et al.*, 2013).

Late risk factors of mortality	Weight
Shock	3
Acute gastrointestinal bleeding	2
Pneumonia	2
Nadir hemoglobin in hospital ≤7.0 g/dl	1
Septicemia	1
Worsened congestive heart failure	1
Neurological complications (e.g. stroke and hypoxic encephalopathy)	1

Hamilton AMRS groups (Table 4.6). This permits monitoring response to therapy and adjustment of overall mortality risk probability (Beliaev, 2013).

There is no high quality evidence in regards to the optimal dosing regimen of EPO for treatment of severe anemia in JW patients. Some experts recommend a very high dose regime of EPO up to 40,000 IU/d (Posluszny and Napolitano, 2014).

RPI can be used to monitor bone marrow response to anemia and EPO administration. RPI can be calculated using Equation 4:

$$\text{RPI} = \text{Retic Count} \times \frac{1}{2} \times \frac{\text{hemoglobin (observed)}}{\text{hemoglobin (normal)}} \quad (4)$$

Hematology consultations can be helpful for a dosing of EPO advice and monitoring bone marrow response in patients with anemia of chronic kidney disease.

Severely anemic JW patients may develop infective complications: septicemia, pneumonia, urinary tract infection, intravenous line-related cellulitis and surgical site infection are reported (Beliaev *et al.*, 2012a). Routine procedures should be followed with a high index of suspicion, early septic screen, and use of antimicrobial therapy as directed by clinical condition and microbiology results. Input from microbiologists and infectious diseases specialists may be useful in early identification of micro-organisms and optimization of antimicrobial therapy.

Clinicians should constantly monitor anemic JW patients' treatment for the development of anemia-related complications, late mortality risk factors, and may use the Hamilton AMRS as a guide to adjust mortality risk during admission.

For JW patients with a significant acute blood loss and severe anemia, the early involvement of critical care physicians to coordinate care and an ongoing multidisciplinary approach is

Table 4.6. Absolute and relative risk of mortality in severely anemic Jehovah's Witness patients according to their Hamilton anemia mortality risk score (AMRS) (modified from Beliaev *et al.*, 2013).

Hamilton AMRS groups	Absolute risk of mortality (%)	Relative risk of mortality	*P*-value
0-2	4	1	–
3-4	29	4.9	0.006
5	40	6.7	0.009
≥6	67	11.2	<0.0005

recommended. Patients in the ICU should be managed with clear directives as to acceptable therapies, and with vigilant blood conservation strategies. Blood testing should be carried out only as required, tests grouped to minimize discard events, and the use of small volume collecting tubes is suggested to reduce waste. It is important for the entire ICU team to be aware of the goals of blood conservation and the concept that every milliliter of blood makes a difference. A restrictive rather than liberal approach to fluid resuscitation may be both necessary and beneficial for JW patients, to avoid excessive hemodilution. The early institution of inotrope and vasopressor support rather than fluid therapy to achieve hemodynamic goals may be helpful. It is vital during the JW patient's course in ICU that the patient's directives as to the use of blood and products are strictly adhered to. Additionally, it is important to appreciate that a competent patient may alter their directives during a course of treatment. Importantly, several studies have demonstrated outcomes equivalent to non-JW patients in JW patients undergoing major surgery (Bhaskar *et al.*, 2010; Pattakos *et al.*, 2012).

Professional interactions with Jehovah's Witness patients

The professional relationship between the doctor and the mentally competent JW patient is patient-centered. It is the clinicians' responsibility and obligation to fully inform patients of the risks and benefits of allogeneic blood and blood products transfusion, but it is the patient's responsibility and right to determine whether to accept or refuse blood transfusion at any stage of their treatment. A multi-disciplinary approach in the management of severely anemic JW patients is advocated (Shander *et al.*, 2014). In this approach clinicians from different specialties work as a team, and plan and implement JW patients' treatment. Importantly, this approach should not undermine the leadership role of a responsible clinician, who must establish an early effective professional relationship with a JW patient and his/her family and maintain it during the entire course of treatment.

Clinicians providing treatment to JW patients may face ethical dilemmas during management. First, the clinician may have difficulty adhering to the patient's health-related values and at the same time achieving the best medical outcome for the patient. The interpretive model of the professional relationship between clinicians and JW patients focuses on utilization of patients'

values, even if patients' decisions may negatively influence medical outcomes (Beliaev, 2010). In the interpretive model, JW patients' religious beliefs, health-related preferences and values are respected and validated. Second, a paucity of healthcare resources may exist, with a resultant inability to provide all high risk patients with high dependency care, such as treatment in an ICU or HDU. In such an environment, severely anemic JW patients, refusing conventional therapy with blood transfusion, may compete with other patients for ICU or HDU beds. In this instance, the ethical principle of respect for patient's autonomy clashes with the principle of formal justice, which states 'equals ought to be treated equally' (Beauchamp, 2001).

In the authors' institution, JW patients scheduled for an elective surgical operation meet a surgeon and an anesthetist in the weeks prior to planned surgery, at a preadmission outpatient clinic. Patient's extended family members are invited to attend the same clinic appointment. During this first meeting the risks and benefits of the planned surgery, anesthesia, blood transfusion and its alternatives are discussed and clearly documented in the patient notes and on the consent to treatment form. Clinicians should not challenge JW patient's religious beliefs, but provide objective information about the risks of bleeding, blood loss, and mortality with acute anemia (Carson and Patel, 2014). It is vital that information about blood conservation strategies be discussed also, including cell saver use, and the venesection and use of autologous blood for re-transfusion. It is important to explore barriers for acceptance of blood transfusion during the first meeting and to clarify whether the patient wishes to discuss this issue again should the risk to life be exceptionally high. An honest and caring attitude toward the JW patient may allow the patient and his/her family members to fully explore all alternatives, and may potentially save lives.

4.4.4 Management of severe anemia with blood transfusion

ARBC transfusion is indicated in hemorrhagic shock, ongoing bleeding with hemodynamic instability, acute hemorrhage with inadequate oxygen delivery, in hemodynamic stable critically ill patients with Hb <7.0 g/dl (Level 1 evidence) (Napolitano *et al.*, 2009).

ARBC transfusion should be considered in critically ill patients requiring mechanical ventilation, resuscitated critically ill trauma patients, in critically ill patients with stable cardiac disease provided they have Hb <7.0 g/dl (Level 2 evidence) (Napolitano *et al.*, 2009).

In hemodynamically stable patients with cardiovascular disease ARBC transfusion should be considered at Hb ≤8.0 g/dl or for symptoms, including chest pain, orthostatic hypotension or tachycardia unresponsive to fluid resuscitation, or congestive heart failure) (Level 2 evidence) (Carson *et al.*, 2012).

To avoid overtransfusion it is recommended that one unit of ARBC to be administered to patients without ongoing bleeding (Level 2 evidence) ((Napolitano *et al.*, 2009). Massive hemorrhage with shock or trauma-induced coagulopathy requires massive blood transfusion, which is defined as acute replacement of more than 50% of patient's EBV per hr or total replacement of the patient blood volume in less than 24 hrs. Slight variations exist in institutional massive transfusion

protocols, but generally include administration of ARBC and FFP in 1:1 ratio followed by the early use of platelets and cryoprecipitate (Ogura *et al.*, 2015).

4.4.5 Management of anemia of critical illness

Critically ill patients are managed with strategies to minimize blood loss, and optimize hematopoiesis. Thus, minimization of blood sampling, use of small volume tubes for samples, closed circuit systems allowing return rather than discarding of blood withdrawn, and early hemostasis following procedures, surgery or trauma are important. Maintenance of complete nutrition and avoidance of infection are routine. Some management strategies, such as the use of EPO, iron therapy, and red blood cell transfusions, while common, remain controversial. Cessation of anticoagulant, antiplatelet, or non-steroidal anti-inflammatory drugs if practicable is desirable. Prevention of gastrointestinal bleeding with the use of gastric acid suppressants may also be helpful.

The use of EPO has not consistently showed a benefit in reducing anemia or transfusion rates in the severely anemic, and concerns in regard to the reported association with increased rates of thrombotic events in ICU suggest that further high quality trials are needed before use of EPO can be recommended (Corwin *et al.*, 2007; Corwin *et al.*, 2002).

Iron therapy has thus far not been well studied in the critically ill. Whilst a state of iron deficiency, or relative iron deficiency, often exists amongst the critically ill, concerns surrounding infections and responses to infections remain. It is thought that the normal physiological response of reducing serum iron in acute illness is protective against microbes, and that supplementation of iron may increase infections. However, there is no high quality evidence to support the beneficial effects, or the potential harmful effects of iron supplementation at present. Further clinical trials are underway at present to determine the role of iron supplementation in critical illness (Litton *et al.*, 2014; Pieracci *et al.*, 2014).

The use of red blood cell transfusions as therapy for severe anemia is common in critically ill patients, but is also subject to much debate. The optimum Hb concentration to maintain in critically ill patients is not clear, and is likely to differ between individual patients and groups of patients, according to clinical condition and co-morbidities, rather than a 'one size fits all' approach. Concerns around the risks of blood transfusion include infection risks, transfusion related acute lung injury, transfusion related immuno-modulation, volume overload, iron overload, and cost implications of a scarce resource. The use of red blood cell transfusions has also consistently been associated with worsened outcomes in critically ill patients, however whether anemia or transfusion is 'worse' for an individual patient remains unclear. The transfusion requirements in a critical care trial in 1999 compared the use of a liberal (transfusion trigger <10 g/dl Hb) to a restrictive (trigger <7 g/dl) transfusion approach in 838 critically ill patients. The use of a restrictive strategy was as effective as a liberal strategy, with the exception of patients with acute myocardial infarction and unstable angina (Hebert *et al.*, 1999). The findings of this study were consistent with several subsequent studies in heterogeneous groups of critically ill patients,

and a restrictive strategy to transfusion in the critically ill became a widely accepted practice. However, several subsequent studies have failed to demonstrate clinical benefits with a restrictive approach and some studies have reported better outcomes in those patients transfused with a liberal approach (Holst *et al.*, 2014; Murphy *et al.*, 2015; Rivers *et al.*, 2001). Possible reasons for these conflicting reports are the use of large heterogeneous groups, and rigid single triggers, such as Hb concentration – where some patients may benefit and others may suffer harm, the overall results of the study are likely to reflect no difference. For any individual, however, a multitude of factors may mean the risks of transfusion outweigh the risks of anemia, or vice versa. Currently, a balanced approach assessing the patient's clinical condition, co-morbidities and susceptibility to potential harmful effects of therapy is recommended, with the aim of providing appropriate therapy, but avoiding unnecessary transfusion.

4.5 Anemia outcomes

Outcomes of acute severe anemia in hospitalized patients are largely dependent on whether patients were treated with blood transfusion or not. Retrospective studies of JW patients, who refused blood transfusion on religious grounds, have reported the odds of death of severely anemic JW patients are low when Hb was 7.1 to 8.0 g/dl, but increase two to three times for every 1.0 g/dl postoperative Hb decrease below 7.0 g/dl (Carson *et al.*, 2002; Shander *et al.*, 2014). At postoperative Hb level from 5.0 to 4.0 g/dl, mortality exceeds 30% percent (Carson *et al.*, 2002).

Another group investigated the mode of death of JW patients with severe anemia (Tobian *et al.*, 2009). The five most common causes of death were bleeding (39%), respiratory failure (39%), renal failure (28%), sepsis (21%), and myocardial infarction (13%). Other causes of death were cardiac arrhythmias, congestive heart failure, pulmonary embolism, pneumonia, other infection, bowel infarction, anoxic encephalopathy and hypovolemic shock (Tobian *et al.*, 2009).

In one study, severe acute and chronic anemia in JW patients was associated with 20% mortality, compared with 2% mortality for severe anemia treated with ARBC transfusions in matched non-JW patients (Table 4.7) (Beliaev *et al.*, 2012a). In the same study, anemic JWs had higher rates of infective complications, including pneumonia, urinary tract infections and intravenous line-related cellulitis (Table 4.7), and longer length of hospital stay, higher rate of hospital readmission and a significantly higher health-care provider expenditure (Beliaev *et al.*, 2012a).

Compared to severely anemic JW patients, who were treated with blood alternatives, ARBC transfusion in non-JW patients was associated with a 94% reduction in mortality. The number needed to treat to prevent death was six. Also, ARBC transfusion decreased cardiac arrhythmia by 96%, shock by 88%, angina by 86%, ischemic myocardial injury by 81%, gastro-intestinal bleeding by 81%, neurological complications by 92%, delirium by 76%, syncopal episodes by 95%, and infective complications by 81% (Beliaev *et al.*, 2012a). ARBC transfusion was cost-effective; its incremental cost-effectiveness ratio in 2011 was US$ 22,515 for each death prevented (Beliaev *et al.*, 2012a).

Table 4.7. Mortality and complications in severely anemic Jehovah's Witness (JW) patients vs. allogeneic red blood cell (ARBC-) transfused patients (modified from Beliaev *et al.*, 2012a).

Complications	JW patients (n=103), n (%)	ARBC-transfusion patients (n=103), n (%)	Odds ratio[1]	95% confidence interval	*P*-value[2]
Mortality	21 (20.4)	2 (1.9)	0.06	0.01-0.30	0.0005
Hemorrhage	27 (26.2)	10 (9.7)	0.40	0.17-0.92	0.031
Gastrointestinal bleeding	11 (10.7)	3 (2.9)	0.19	0.05-0.78	0.021
Shock	22 (21.4)	3 (2.9)	0.12	0.03-0.46	0.002
Infective complications	68 (66)	28 (27.2)	0.19	0.10-0.37	0.0005
Pneumonia	28 (27.2)	4 (3.9)	0.11	0.03-0.33	0.0005
Urinary tract infection	26 (25.2)	11 (10.7)	0.30	0.13-0.68	0.004
Intravascular line/related cellulitis	15 (14.6)	1 (1)	0.05	0.01-0.43	0.006
Cardiac arrhythmia	15 (14.69)	1 (1)	0.04	0.01-0.37	0.004
Angina	15 (14.6)	4 (3.9)	0.14	0.04-0.49	0.002
Ischemic myocardial injury	17 (16.5)	6 (5.8)	0.19	0.06-0.57	0.003
Heart failure	27 (26.2)	9 (8.7)	0.21	0.08-0.55	0.001
Stroke/hypoxic encephalopathy	9 (8.7)	1 (1)	0.08	0.01-0.68	0.021
Acute/acute on chronic renal failure	35 (34)	17 (16.5)	0.34	0.16-0.72	0.005
Delirium	16 (15.5)	6 (5.8)	0.24	0.08-0.72	0.011
Depression	7 (6.9)	1 (1)	0.09	0.11-0.80	0.031
Syncope	17 (16.5)	1 (1)	0.05	0.01-0.44	0.007

[1] Logistic regression adjusted for age, European ethnicity, Maori ethnicity, diabetes mellitus, bronchiectasis/tuberculosis, acute/chronic renal failure.
[2] $P<0.05$.

Anemia in critical illness is consistently associated with worse outcomes, including increased mortality, hospital and ICU length of stay, and morbidity (Corwin *et al.*, 2004). It is not clear whether this is entirely a causative effect, or a marker of worse illness severity.

Chronic anemia, particularly iron-deficiency anemia, is one of the leading global risk factors of death. Pregnant and postpartum women, and young children are the most commonly affected by iron deficiency because of the high iron requirements of pregnancy and infant growth. Approximately 20% of perinatal mortality and 10% of maternal mortality in developing nations is attributable to iron deficiency (WHO, 2002). In total, iron deficiency is an attributable cause of 0.8 million (1.5%) of deaths globally and accounts for the loss of approximately 35 million healthy life years (2.4% of global disability-adjusted life years (DALYs)) (WHO, 2002). Iron deficiency impairs mental development and is the cause of mild mental retardation. It decreases

aerobic work capacity and fitness through impaired oxygen transport and muscle respiratory efficiency as well.

Pernicious anemia is associated with tonsillar cancer, esophageal squamous cell carcinoma, gastric adenocarcinoma and carcinoid tumours, intestinal and liver cancers, myelodysplastic syndrome, acute myeloid leukemia and myeloma (Murphy *et al.*, 2015a). Folate deficiency increases likelihood of neural tube defects development during pregnancy and is associated with preterm delivery, fetal growth retardation and low birth weight (Bailey *et al.*, 2015).

Chronic kidney disease anemia is associated with a progressive decline in renal function, development of left ventricular hypertrophy, left ventricular dilatation and an increased likelihood of death from cardiovascular disease (Rao and Pereira, 2005).

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Vitamins and other small compounds in the therapy of blood diseases

5. Vitamins C and E supplementation in sickle cell anaemia

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Abstract

Oxidative stress is the group of cellular processes that produce reactive oxygen species. Divalent metals as iron can catalyse oxidative reactions and haemoglobin, which contains iron, generates large amounts of oxidants. There is a normal physiologic rate of reactive oxygen species generation and clearance; however, in some pathologic conditions like haemolytic anaemias, there is an unbalance in this system. Erythrocytes of sickle cell anaemia patients continuously produce larger amounts of pro-oxidants than normal cells, and oxidative stress seems to play a significant role in the pathophysiology of the disease. In erythrocytes, oxidative stress primarily affects the membrane and results in haemolysis. There is evidence that the combination of vitamins C and E reduces the generation of pro-oxidants and markers of cellular oxidative damage in both normal individuals and in patients with various diseases. It was postulated that its use in sickle cell anaemia patients could prevent adhesion and phagocytosis of oxidized erythrocytes and consequently haemolysis. This could bring benefits by reducing anaemia, clinical complications and improving the quality of life of these patients.

Keywords: haemolysis, tocopherol, ascorbic acid, oxidative stress, antioxidants

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Key facts

- Sickle cell anemia is a common hereditary blood disorder characterized by an abnormality in the hemoglobin structure. This leads to a propensity for the erythrocyte to assume an abnormal, rigid, sickle shape under low oxygen tension.
- Continuous changes of erythrocytes makes them irreversibly sickled. These cells can cause vaso-occlusion of the microvasculature and are subject of intravascular hemolysis and phagocytosis in the reticuloendothelial system.
- Sickle cell anaemia (SCA) is associated with acute and chronic complications as crises of severe pain, anemia, infections, priapism, stroke, retinopathy, osteonecrosis, and its carriers have an increased risk of death.
- Professionals involved in the management of SCA patients should seek new ways to manage them, to mitigate the disease complications, allowing them to have a life as close to normal as possible.

Summary points

- There is evidence that oxidative stress has a role in maintenance or worsening of hemolysis in hemolytic anemias and could have a central role in the pathophysiology of sickle cell disease.
- SCA patients have overtly deficiency of antioxidant vitamins C and E.
- There is *in vitro* evidence of reduction of oxidative stress when using antioxidants *in vitro* in patients with hemolytic anemias, including SCA, but *in vivo* evidence of benefit is lacking.
- Supplementation with vitamins C and E do not improve anemia and may increase markers of hemolysis and inflammation in SCA patients. Currently, supplementation cannot be recommended in this context.

Abbreviations

AGE	Aged garlic extract
ALA	α-lipoic acid
CBC	Complete blood count
GSH	Glutathione
HbA	Haemoglobin A
HbS	Haemoglobin S
LDH	Lactate dehydrogenase
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NSAIDs	Nonsteroidal anti-inflammatory drugs
O_2^-	Superoxide radical
RDA	Recommended dietary allowance
SCA	Sickle cell anaemia
UL	Tolerable Upper Intake Level

5.1 Introduction

SCA is a very common inherited disease, caused by the presence of a point mutation (substitution of adenine by guanine) in the sixth codon of the β chain of haemoglobin gene, which replaces valine for glutamic acid in the outer surface of β chain (Sommer *et al.*, 2006). When it is inherited in homozygosis, there is complete absence of normal β chains, and the consequent formation of HbS, consisting of two chains of normal α globin plus two chains of mutant S β-globin (Hebbel, 2005; Smith and Scherer, 2010). SCA has chronic evolution with great potential to cause morbidity and mortality, and worsen the quality of self, social, family and reproductive life, in addition to overload the health system (Smith and Scherer, 2010).

Traditional pathophysiology concepts of SCA attach all its clinical features to the HbS, which has low solubility and a tendency to polymerization when deoxygenated, generating low deformability of erythrocytes containing polymers, sickling, and occlusion of the microvasculature with sickled cells. Besides, continuous changes of morphology cause chronic injury to the membrane and an irreversibly sickled erythrocyte. These cells, which can represent up to 40% of erythrocytes in SCA patients, have short half-lives and are quickly cleared, one third by intravascular haemolysis and two thirds by phagocytosis in the reticuloendothelial system (Sauntharajah, 2005). Intravascular haemolysis fleeces nitric oxide, the main mediator of vascular muscle relaxation (Rother *et al.*, 2005). Nitric oxide bioavailability reduction and the increased expression of several known vasoactive molecules have a central role in endothelial dysfunction (Aslan and Freeman, 2007; Sauntharajah, 2005)

Sickling, haemolysis, endothelial adhesion and dysfunction and inflammation pathways activation: these factors together account to a greater or lesser degree for the chronic anaemia, recurrent painful episodes, retarded growth and development, recurrent infections, neurological, pulmonary, renal, hepatobiliary, ocular, cardiac, and bone complications, leg ulcers and priapism observed in SCA patients. Despite this, polymerization continues to be the *sine qua non* event, a contemporary understanding of the pathophysiology requires perspective derived from biophysics, biochemistry, molecular and cellular biology, genetics and coagulation (Sauntharajah, 2005)

Besides being less soluble and having abnormal electrophoretic mobility, HbS is also unstable (Klings and Farber, 2001). It has been shown that sickle erythrocytes produce more amounts of oxygen radicals than normal cells (Hebbel *et al.*, 1982; Repka and Hebbel, 1991). Physiological oxygenation and deoxygenation processes of haemoglobin generating methaemoglobin (self-oxidation) are more pronounced in SCA (Sauntharajah, 2005). This process usually is followed by the generation of small amounts of O_2^-; however, as HbS is 1.7 times more self-oxidized than HbA, more O_2^- is produced in this context (Amer *et al.*, 2006; Hebbel *et al.*, 1982; Misra and Fridovich, 1972). In addition, there are haemicromos formations that release iron, with denaturation of HbS and Heinz bodies' precipitation (Welch *et al.*, 2002).

Consumption of antioxidant defence mechanisms enhances other oxidative lesions. Free haem inhibits various enzymes required for the generation of NADH and NADPH (Hebbel *et al.*, 1982) and there is activity reduction of the hexose-monophosphate shunt, and of GSH concentration inside sickled cells (Lachant *et al.*, 1983). Free oxygen radicals attack membrane lipids and proteins, catalyse lipid hydroperoxides and produce alkoxyl and peroxyl radicals (Becker *et al.*, 2004). These radicals increase the damage from new cycles of lipid peroxidation. The perpetuation of oxidative stress changes, beyond the state of lipids and membrane proteins, the integrity of HbS molecule and the erythrocyte metabolism (Hebbel, 1982, 2005). Continuous oxidative stress can also promote the expression of molecules that increase the adherence to endothelial cells, activation of inflammation and coagulation system and the recognition of cells by macrophages, enhancing extravascular haemolysis (Sauntharajah, 2005).

Vaso-occlusives crises could be affected by increased oxidative stress. They result from abnormal relations between normal and sickled erythrocytes, neutrophils, platelets, endothelium and vasoconstriction in areas of poor perfusion and reperfusion. Situations of reperfusion (Dhalla *et al.*, 2000; Klings and Farber, 2001; Leo *et al.*, 1997) and neutrophils binding or activation by endothelium (Hofstra *et al.*, 1996; Mohamed *et al.*, 1993) increases the oxidant radical levels. Besides, oxidative stress may play a role in microvascular dysfunction and development of extensive organ damage seen in these patients (Wood and Granger, 2007).

It is well known that several of the molecular lesions caused by oxidative stress can be reduced by *in vitro* antioxidants (Amer *et al.*, 2006). Vitamin E, vitamin C, NAC and other antioxidants reduce tissue damage from free radicals imprisonment and deactivation of reactive oxygen molecules, reducing net oxidative stress (Sesso *et al.*, 2008). Trials showed inhibition of the formation of irreversibly sickled erythrocytes and dense erythrocytes as well as increase in the

intracellular GSH concentration in HbS cells treated with NAC *in vitro.* Reduction of vaso-occlusive events among patients treated with this antioxidant *in vivo* was also observed (Pace *et al.*, 2003). Supplemental vitamin C showed oxidative damage protection of erythrocytes treated *in vitro* with hydrogen peroxide (Lachant and Tanaka, 1986). Vitamin E supplementation reduced the percentage of irreversibly sickled cells (Natta *et al.*, 1980).

Trials evaluating serum levels of vitamins and trace elements showed a significant reduction of the levels of vitamins A, C and E in patients with SCA when compared to normal controls (Chiu *et al.*, 1990; Hasanato, 2006; Ray *et al.*, 2007). These results, added to the outcomes of antioxidant treatments of SCA cells *in vitro*, suggest a rationale for their *in vivo* use in SCA patients (Amer *et al.*, 2006).

5.2 Vitamin E

Vitamin E (α-tocopherol) chemical and biological aspects have been intensive studied for over 50 years, and there is available evidence demonstrating conclusively that its main physiological role is tissue protection against destructive and undesirable oxidation (Amer and Fibach, 2004). Although other functions are being studied, the antioxidant function is by far the best documented (Pryor, 2000). Tocopherol is a potent antioxidant, capable of breaking chains of free radicals, and may have effect reducing oxidative stress *in vivo* (Pfeifer *et al.*, 2008). Its ability to prevent propagation of oxidation of polyunsaturated fatty acids of cell membranes (Spielberg *et al.*, 1979) and to reduce oxidative stress in lipids *in vivo* is also already demonstrated (Das *et al.*, 2004). The big question that has been discussed in the past 20 years is that if supplementation is capable of reduce harmful oxidative effects in healthy subjects and patients with chronic diseases. Results from clinical studies conducted so far are conflicting.

Vitamin E supplementation reduced laboratory markers of platelet activation in non-insulin-dependent diabetes (which is persistently observed in the population), with similar effect to that seen with optimized glycaemic control (Davi *et al.*, 1999). Supplementation of smokers for twenty weeks with escalating doses reduced the susceptibility of erythrocytes to undergo lipid peroxidation mediated by hydrogen peroxide in a dose-dependent manner (Brown *et al.*, 1997). A study of 65 β-thalassemia children observed low baseline serum levels of vitamin E; supplementation normalized serum Vitamin E levels and reduced markers of haemolysis mediated by hydrogen peroxide, with however, no change in haematocrit (Suthutvoravut *et al.*, 1993). A trial with nine intermediate β-thalassemic patients undergoing treatment with vitamin E for three months showed significant reduction of reactive oxygen species in erythrocytes measured by flow cytometry, increased erythrocyte GSH levels, and reduction in circulating reticulocytes, without changes in anaemia or other haematological parameters (Pfeifer *et al.*, 2008). No studies have reported significant side effects.

One mg of vitamin E corresponds to 1.49 IU of α-tocopherol (and 1 IU corresponds to 0.67 mg of vitamin E). The daily dietary recommendation (RDA), the dietary dose of a compound that

is sufficient to meet the nutritional needs of 98% of the normal population of a certain age and gender, is 15 mg/d (22 IU of α-tocopherol), for adult men and women (Institute of Medicine, 2000). Daily administration of 200-600 mg (300-900 IU) is innocuous for most individuals (Bieri *et al.*, 1983). There is a consensus among experts that the drug is safe up to 800 UI/d (530 mg/d), and probably safe with twice this dosage (1,600 IU/d: 1,060 mg/d) (Pryor, 2000).

UL is the largest daily intake dose of a compound that brings no risk of adverse effects on the absolute majority of individuals in general population. The UL for vitamin E was established at 1000 mg/d by the Food and Nutrition Committee of the American Institute of Medicine because of an increased tendency to hemorrhage with higher doses (Institute of Medicine, 2000). Extensive review of tolerance, toxicity and safety of vitamin E use was concluded that doses up to 1,200 IU/d (800 mg/d) are generally not associated with adverse effects (diarrhoea, nausea, flatulence, abdominal cramps, creatinine ratio, coagulation disorders), that usually appear at doses above 1,500 IU/d (1000 mg/d), and are reversible after withdrawal (Kappus and Diplock, 1992). There is no information of acute overdose in humans (Micromedex, 2010b).

5.3 Vitamin C

Vitamin C (ascorbic acid) is a biological reducer agent for a variety of oxidants, one of the most important intracellular water-soluble antioxidants. However, it is not the unique antioxidant available in its action site; there are other intracellular soluble antioxidants available, as uric acid, and GSH. In addition, vitamin C, unlike vitamin E, is an essential enzymatic cofactor and has several other well-established biological functions.

In vitro human plasma studies show that vitamin C is the first line of defence against lipid peroxidation induced by a variety of oxidants (McCall and Frei, 1999). Normal erythrocytes treated with vitamin C show potent reduction of methaemoglobin formation and maintenance of GSH reductase levels, reversing some of the oxidant actions caused by *in vitro* oxidants (Krukoski *et al.*, 2009). There is evidence of reduction of lipid oxidative damage in smokers and non-smokers subjected to supplemental vitamin C (McCall and Frei, 1999). Two clinical studies conducted in Africa noted a reduction in the proportion of irreversibly sickled cells and increased concentration of haemoglobin and haematocrit in adults (Jaja *et al.*, 2002a) and children (Jaja *et al.*, 2002b) with SCA who underwent low-dose supplementation of vitamin C.

The absorption of vitamin C is highly dose-dependent and individuals already saturated through their diet will excrete excess and probably will not benefit from supplementation (Lykkesfeldt and Poulsen, 2009). However, definition of the optimal serum level of vitamin C serum still raises controversy. The RDA of vitamin C for adults is 75 mg/d for women and 90 mg/d for men, a dose that maintains maximum concentration of ascorbate in neutrophils with low urinary excretion (Institute of Medicine, 2000). Furthermore, individuals with increased demands have lower serum levels, probably due to increased consumption (Carr and Frei, 1999; Lykkesfeldt and Poulsen, 2009). Thus, the daily requirement of this vitamin would be dependent on the oxidative

status of the individual. It has been observed that the serum levels of vitamin C in patients with SCA are lower than in controls (Hasanato, 2006; Ray *et al.*, 2007).

Ascorbic acid administered orally is generally well tolerated. The UL for vitamin C for adults is 2,000 mg/d, based on the tendency to develop osmotic diarrhoea (Institute of Medicine, 2000). Doses greater than 3,000 mg/d can cause polyuria, a light osmotic diarrhoea and other gastrointestinal abnormalities, and may result in hyperoxaluria and renal stone formation. There is no information of acute overdose in humans (Micromedex, 2010a).

Most clinical trials evaluating antioxidant vitamins used much higher doses than the recommended daily intake, close to the UL. Despite the recommendation against use of doses near or above the UL for the general population, these high doses may be appropriate for research in controlled clinical trials, with the aim of showing an antioxidant benefit in supplementation (Institute of Medicine, 2000). Although their safety is relatively well defined, there are many issues to be resolved regarding the efficacy of supplemental antioxidant vitamins for the prevention of oxidative damage (McCall and Frei, 1999), both in patients and in general population.

Understanding the activity of reactive oxygen species, including their synergistic activity, reactivity, short half-life, continuous production next to biological targets and ability to change into even more reactive species suggests that antioxidants should be administered in combinations, and should be prepared to clear both reduced and oxidized forms, without transition metals in their formulations (Fibach and Rachmilewitz, 2008). There is evidence that the combination of vitamins C and E reduces oxidative stress markers *in vivo* alone (Naziroglu *et al.*, 2010a,b; Rizzo *et al.*, 2008; Rodrigo *et al.*, 2008; Ruiz-Ramos *et al.*, 2010) or in combination with other drugs (Castillo *et al.*, 2010; Girodon *et al.*, 1997; Hemila and Kaprio, 2011; Howard *et al.*, 2002), both in normal individuals and in patients with various diseases. Vitamin E at a dose of 400 IU (266 mg/d) already has antioxidant effects *in vivo* and vitamin C is important in recycling tocopheryl radical, which justifies the use of these compounds together (Kontush *et al.*, 2001; Talaulikar, 2010).

5.4 Vitamins C and E and sickle cell anaemia

Until 2012 there were ten trials of antioxidant administration to SCA patients listed in PubMed:

- Natta *et al.* (1980): vitamin E supplementation 300 mg/d for 6 to 35 weeks increased the serum levels of tocopherol and reduced the proportion of irreversibly sickled cells.
- Muskiet *et al.* (1991): administration of zinc 109 mg + vitamin E 460 mg + vitamin C 600 mg + linoleic acid 11 g + linolenic acid 1.5 g (soybean oil) for eight months followed by seven months of vitamin C 600 mg + vitamin E 420 mg + omega-3 rich fatty acids (fish oil) to 13 children with SCA reduced irreversibly sickled cells, but did not change haemoglobin, reticulocytes, LDH, liver enzymes, renal function, white blood cells, number of pain crisis, number of aplastic crises or other clinical complications. The authors concluded that increased

antioxidant status of erythrocytes by the administration of vitamin C and E did not affect the haemolytic component of SCA, and that its beneficial effects are questionable in this context.

- Ohnishi *et al.* (2000): AGE administration 6 g + vitamin C 6 g + vitamin E 800 mg for six months vs. placebo to 20 patients with SCA in a non-randomized and non-blinded trial increased haematocrit and reduced the average number of painful attacks per individual (without specifying the criteria for the definition of 'painful crisis') in the antioxidant group compared to the placebo group during the study period. Other CBC parameters and laboratorial evidence of haemolysis have not been studied.
- Jaja *et al.* (2002a): vitamin C 100 mg/d for six weeks to 15 children (4-11 years old) increased haemoglobin, haematocrit, the proportion of foetal haemoglobin, reduced irreversibly sickled cells and increased resistance to osmotic lysis of erythrocytes.
- Pace *et al.* (2003): NAC 2,400 mg to five SCA patients with a history of at least two pain crises per year and 6% dense cells for seven months vs. three patients in placebo, in a randomized and double-blind way, increased levels of erythrocyte GSH and reduced pain crisis episodes. No studies of CBC and haemolysis were reported. The authors suggested the need for a phase III study to define the NAC efficacy and safety in long-term for these patients.
- Okpala *et al.* (2004): omega-3 eicosapentaenoic acid (EPA 15 mg/kg/d) and docosahexaenoic acid (DHA 10 mg/kg/d) to 16 SCA patients for six months reduced serum indirect bilirubin and the median number of painful crises, with no change in haemoglobin, neutrophils, platelets and serum interleukin-6.
- Jaja *et al.* (2005): vitamin E 100 mg/d for six weeks to ten SCA children (4-10 years old) increased haemoglobin, haematocrit, percentage of foetal haemoglobin, cell resistance to osmotic lysis and decreased irreversibly sickled cells. No haemolysis parameters were reported.
- Bao *et al.* (2008): zinc 75 mg/d for three months vs. placebo (randomized, double-blind) to 36 SCA patients resulted in increased plasma zinc levels and total antioxidant capacity, reduction of plasma nitrite and nitrate, lipid peroxidation products, DNA oxidation products and change in expression of proteins associated with inflammation and endothelial dysfunction in supplemented patients. Furthermore, there was reduced incidence of infections episodes, with no difference in the incidence of pain crisis; and increased erythrocytes, haemoglobin level and haematocrit pre vs. post-intervention in the zinc group without modification in placebo group. Further CBC parameters did not change in either group. No information of reticulocyte count or other haemolytic laboratorial parameters were reported.
- Martins *et al.* (2009): non-randomized, non-blind supplementation of ALA 200 mg/d vs. placebo for 20 SCA patients for three months reduced GSH peroxidase activity but did not affect other oxidation markers (activity of catalase, superoxide dismutase levels malondialdehyde or carbonyl, total antioxidant capacity) or clinical parameters (erythrocytes, haemoglobin, haematocrit, reticulocytes, ferritin, transferrin). No other markers of haemolysis were studied.
- Jaja *et al.* (2008): vitamin C 300 mg/d for six weeks to ten SCA patients modified electrocardiographic parameters; CBC or haemolysis markers were not reported.

These trials are highly heterogeneous, have different designs, evaluated different outcomes and different antioxidants. Only four of them studied vitamin C and/or E, alone or in combination with other drugs. Two supplemented vitamin C (Jaja *et al.*, 2002a, 2005) in low doses (100 mg)

for short periods (six weeks), with improvement in the mean haematocrit and haemoglobin. One should note that the initial haemoglobin was extremely low (4.1±0.5 g/dl pre-intervention × 6±0.6 g/dl post-intervention), even for SCA patients. Since the trial was not placebo-controlled, this outcome is of little clinical value. The third trial evaluated vitamins C and E in combination with AGE (Ohnishi *et al.*, 2000) with very high doses of vitamin C (6 g), impractical in clinical practice. In addition, the authors made clinical assumptions in a non-blinded study (change in the frequency of painful crises and well-being), and the laboratorial evaluation consisted only of haematocrit, an unreliable anaemia marker. The fourth trial, way better designed than previous ones, evaluated vitamins C and E at intermediate doses for fifteen months in association with zinc + soybean oil + fish oil, and found no difference in anaemia markers, haemolysis and painful crises.

In 2013, Arruda *et al.* (2013) published a randomized, double-blind, placebo-controlled trial to evaluate the impact of vitamins C 1,400 mg and E 800 mg/d vs. placebo orally for 180 days in 83 SCA patients. There were no significant baseline differences between the two groups regarding clinical complications or laboratorial tests. Sixty percent of the patients were overtly vitamin C deficient, 70% were vitamin E deficient. Supplementation significantly increased and normalized serum levels of both vitamins. The expected outcomes were reduction of haemolytic markers (with the consequent improvement in anaemia) and of acute complications of the SCA in the intervention group, considering that haemolysis, anaemia and vaso-occlusion are, at least to some extent, caused or aggravated by oxidative stress. However, no significant changes in haemoglobin levels and other anaemia markers and clinical complications were observed, and, unexpectedly, there were a significant increase in all haemolytic markers (reticulocyte count, haptoglobin, indirect bilirubin, lactate dehydrogenase, aspartate aminotransferase, uric acid) and some inflammatory markers (neutrophil count and C-reactive protein) with vitamin supplementation, not observed in the placebo group. The exact mechanisms to explain this findings and their clinical significance remain to be determined.

If it were only the lack of benefit, without worsening of any parameter, one could conclude that the used doses were too low to achieve a significant antioxidant effect (Talaulikar, 2010). But since there was a consistent increase of all markers of haemolysis, the high doses of antioxidants used could even explain the negative result.

The trial studied doses considered safe (vitamin C 1,400 mg/d + vitamin E 800 mg/d) (Institute of Medicine, 2000; Kappus and Diplock, 1992; Micromedex, 2010a,b; Pryor, 2000), that were at least 20% less than the UL for both vitamin C (2,000 mg/d) and vitamin E (1000 mg/d) (Institute of Medicine, 2000). Furthermore, no supplemented patient reached serum level above the reference value, indicating that no patient reached toxic levels. Besides, no other toxic effect was observed (renal, hepatic, and clinical). Taken together these data make it difficult to assign the negative outcome to a dose-related toxicity.

SCA patients, even those without overt vitamin C or E deficiency, have lower serum levels of these vitamins than the normal population (Arruda *et al.*, 2013; Chiu *et al.*, 1990; Hasanato,

2006; Ray *et al.*, 2007). One could hypothesize that this could be a protective mechanism. The increase of serum levels to those that would be 'normal' could have deleterious effects, since the 'low' protective levels would be lost with supplementation. Since these 'normal' levels could be achieved with other supplementation in lower doses, their safety should also be further studied before recommending wide supplementation for this population (Sanders *et al.*, 2010).

Another possible explanation for the negative outcome is that it reflected patients with normal antioxidant capacity and/or normal serum vitamin levels before the intervention. These patients would eliminate excess water-soluble vitamins (such as vitamin C) but fat-soluble vitamins like vitamin E may accumulate and supplemented patients could develop complications related to hypervitaminosis. This possibility was already reported for vitamins A, D, and K, however, there is no description regarding vitamin E until now.

To test this hypothesis, the authors evaluated laboratory markers of haemolysis and inflammation in patients receiving vitamins, pre and post supplementation, comparing the group with overt deficiency of the studied vitamins with the group with normal serum levels. There was no baseline test difference, except obviously the median serum levels for both vitamins. After the intervention, patients previously deficient presented more pronounced worsening of haemolysis markers and inflammation (with statistically significant difference between these groups) than patients with normal baseline serum levels.

This data reinforces the previous hypothesis: patients with SCA would have naturally lower serum levels of these vitamins than the normal population and many would be overtly deficient; as deficient individuals were more susceptible to worsening of haemolysis markers and inflammation when supplemented, it is postulated that the decreased bioavailability of these vitamins could be a protective mechanism for these patients, which would be lost with any supplementation.

Moreover, despite evidence showing correlation between oxidative stress and haemolysis, the true cause and effect relationship of this association it is not known. It is possible that the biochemical changes of oxidative stress in SCA may actually have a major role in the pathophysiology of the disease, but not quite as a causative factor. This being the case, reversal of oxidative stress would not be able to reduce haemolysis, anaemia or clinical symptoms and complications of the disease.

Fortunately, there was no worsening of anaemia (haemoglobin and haematocrit) in either group. The results leave open the possibility that this worsening could be noticed if the observation period were extended. Supplementation had no effect on clinical endpoints: chronic use of NSAIDs or opioids, hospitalization for painful crisis, transfusion requirements, priapism episodes, physical symptoms or the self-assessment of well-being and quality of life as measured by objective clinical scores. There were no significant adverse reactions. However, there was a no statistically significant increase in the incidence of acute chest syndrome and two patients experienced ischemic stroke during the observation period in the vitamin group. Despite the small number of patients with these manifestations, which can lead to bias, the evaluation of

the laboratory results allows speculation whether worsening of haemolysis could have had more serious detrimental effects if the intervention period was longer.

The randomized trial provided strong evidence that antioxidant vitamins C and E at high doses, were ineffective in reducing the symptoms and complications in SCA patients, and were involved in significant worsening of laboratory markers of haemolysis and inflammation. These results support the recommendation against their use in this clinical context. Besides, the trial inevitably raises questions about the safety of the use of supraphysiological doses of vitamins C and E for SCA patients. Nonetheless, this unfavourable outcome cannot be extrapolated to lower doses of the same vitamins and other antioxidants drugs, including those including those acquired only in normal diet. The antioxidant-rich diet may be associated with a benefit that is not comparable with pharmacological supplements. The use of each of these vitamins in smaller doses, or other antioxidants drugs for SCA patients could be beneficial, and probably should be evaluated in other clinical trials. Additionally, these findings do not exclude the importance of oxidative stress in the pathophysiology of sickle cell disease; only provide another example of lack of efficacy and possible risk in the use of antioxidants in pharmacological doses in an attempt to reduce symptoms and chronic disease complications.

5.5 Conclusions

Supplementation with vitamins C and E did not improve anaemia and, surprisingly, increased markers of haemolysis and inflammation in SCA patients. The exact mechanisms to explain these findings and their clinical significance remain to be determined. With the knowledge gained so far, supplementation cannot be recommended in this context, even for overtly deficient SCA patients.

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6. Management of anemia by convective treatments and vitamin E coated membranes

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Abstract

Anemia is a common complication in hemodialysis (HD) patients. The retention of uremic toxins may inhibit erythropoiesis and reduced erythrocyte survival. HD treatment has the potential of improving anemia and reduce erythropoietin stimulating agents (ESA) dose. Standard low-flux HD can remove uremic toxins of small molecular weight inhibiting erythropoiesis. Accordingly, anemia improves at the start of HD. However, middle and large molecules can be cleared only by convective treatments. Data from more recent, large, observational or randomized trials using high-flux convective HD did not find a significant improvement in anemia parameters. Given that chronic inflammation and oxidative stress may be involved in anemia pathogenesis, several trials tested the possibility that high-flux HD using a biocompatible, vitamin E-coated membrane may improve anemia. Even if available data are conflicting, many studies suggested a trend towards an increase in hemoglobin levels and/or a reduction in ESA dose needs. The effect seems to be clearer in those patients who are hyporesponsive to ESA treatment. Data from two larger randomized clinical trials have recently questioned the efficacy of vitamin E-bonded membranes on anemia parameters. Altogether, available evidence is insufficient to recommend the use of newer HD modalities just as a therapeutic tool for anemia management.

Keywords: hemodiafiltration, hemodialysis, erythropoietin, chronic kidney disease, oxidative stress

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Key facts

- Anemia is a frequent complication of chronic kidney disease on dialysis patients. Left untreated it may impair quality of life and has been associated with higher mortality and hospitalization risk.
- Among the pathogenic factors causing anemia, the retention of uremic toxins may inhibit erythropoiesis and reduce red blood cell survival. Chronic inflammation and oxidative stress also have a role.
- Thanks to the removal of uremic toxins, the hemodialysis (HD) treatment can improve anemia and reduce the need of erythropoietin stimulating agents (ESA) dosage. The higher the dialysis dose the better is anemia. In well-dialyzed patients a plateau is reached.
- Convective treatments are innovative and ef cient HD modalities that use biocompatible membranes, ultrapure dialysate and high-fluxes. These characteristics reduce inflammation and increase the removal of middle and large uremic toxins.
- Vitamin E-coated membranes have been developed with the idea of combining the positive effects of a biocompatible membrane with the possibility of reducing oxidative stress thanks to the contact of blood with vitamin E during HD.

Summary points

- Dialysis dose has a significant effect on anemia and ESA dose requirements. This has been clearly observed in the early phase of dialysis, when the treatment was not very ef cient.
- Small, uncontrolled studies using high permeable and biocompatible membranes or high-fluxes found anemia improvement.
- Data from more recent, large, observational or randomized trials using high-flux convective HD did not find a significant improvement in anemia parameters.
- Data in the literature are conflicting about the effect of online hemodiafiltration on anemia parameters; it is possible that higher filtration volumes may be more effective.
- In HD patients several clinical trials tested the ef cacy of vitamin E-coated membranes on anemia; ESA resistant patients are more likely to benefit from this dialyzer.

Abbreviations

8-OhDG	8-hydroxy-2´-deoxyguanosine
CKD	Chronic kidney disease
CRP	C-reactive protein
EPO	Erythropoietin
ERI	Erythropoiesis resistance index
ESA	Erythropoietin stimulating agent
Hb	Hemoglobin
HD	Hemodialysis
HDF	Haemo dia filtration
HF	Haemofiltration
IFN-γ	Interferon-γ
IL-1	Interleukin 1
IL-6	Interleukin 6
LDL	Low-density lipoproteins
MDA	Malondialdehyde
OL-HDF	Online haemodiafiltration
RCS	Reactive carbonyl species
TNF-α	Tumor necrosis factor-α

6.1 Introduction

Anemia is a major negative clinical characteristic of patients with chronic kidney disease on dialysis. Many factors other than absolute or relative EPO deficiency may contribute towards causing it. Although absolute or functional iron deficiency is probably the most important factor affecting the response to ESA in most patients, occult blood loss, infection, inflammation and oxidative stress are also of importance. Given that bone marrow suppression may also be present, probably induced by the retention of toxic metabolites, and that HD *per se* can contribute to worsen chronic inflammation and oxidative stress, the more the HD procedure is efficient and biocompatible, the higher is the possibility to improve anemia. Starting from this hypothesis, many studies have been done testing the role of dialysis dose, convective treatments and vitamin E-bounded membranes on anemia. The possibility that innovative HD techniques may improve anemia and decrease the need for ESAs has become of particular interest in recent years. Indeed, data coming mainly from clinical trials testing the role of complete anemia correction with ESA have shown a possible increase in thromboembolic events (in particular stroke) or cancer related death (Pfeffer *et al.,* 2009). The risk seems to be higher when ESA are given at high doses because of patient's hyporesponsiveness (Szczech *et al.,* 2008) possibly related to inflammation and/or oxidative stress. In this perspective, any effort aimed at reducing ESA requirements while obtaining the desired Hb target could be of importance.

6.2 The role of uremic toxins and oxidative stress on anemia

Already in the sixties, toxic substances inhibiting erythropoiesis were found in serum of uremic nephrectomized rabbits (Kuroyanagi and Saito, 1966). Accordingly, it is common observation that anemia often improves at the start of dialysis. During the years, a number of discovered substances have been suggested as possibly implicated in anemia pathogenesis. Some of them act by inhibiting erythropoiesis in the bone marrow, while others are inflammatory mediators generally suppressing erythropoiesis. In addition, some toxins may also reduce erythrocyte survival. Various polyamines, such as spermine, spermidine, putrescine (Kushner *et al.,* 1991) are increased in the sera of HD patients and negatively correlated to erythropoiesis. However, the serum concentration of polyamines is very low and consequently it is difficult to measure their levels. Indeed, some have questioned the specificity of these substances to suppress erythropoiesis (Segal *et al.,* 1988; Yoshida *et al.,* 2006). High-molecular weight inhibitors, which can be eliminated partially only by high-flux dialyzers, have also been found. Many of them are still largely unknown. Interestingly, polyamine-protein conjugates have been shown to selective inhibit colony-forming units-erythroid proliferation (Galli *et al.,* 2001).

Among uremic toxins, those derived from tryptophan metabolism are also of particular interest because of their cardiovascular toxicity (Sallée *et al.,* 2014). These include toxins from the kynurenine and indolic pathways. Quinolinic acid is the product of tryptophan oxidation. *in vitro* and *in vivo* experimental studies have shown that it can inhibit erythropoiesis (Kawashima, 1987) and decrease EPO synthesis (Pawlak, 2003). Indoxyl sulfate is an endogenous molecule derived from indole, a product of protein metabolism by intestinal bacteria, which acts via the aryl hydrocarbon receptor. Indoxyl sulfate can suppress EPO expression through a reduced induction of hypoxia-inducible factor 1 target genes in the presence of hypoxia (Tanaka, 2013).

A concomitant impairment of red blood cell survival may have also a role but has been largely neglected. The 'suicidal' death of erythrocytes, named eryptosis, can be triggered by some uremic toxins, such as vanadate (Lang, 2012), acrolein (Ahmed *et al.,* 2013b) and methylglyoxal (Lang, 2012). Indoxyl sulfate may be also a mediator of enhanced eryptosis in CKD (Ahmed *et al.,* 2013a).

Among inflammatory cytokines, IL-6 has been found to antagonize EPO effect on bone marrow proliferation (Jongen-Lavrencic *et al.,* 1996). Its levels were directly related to ESA dose in HD patients (Goicoechea *et al.*, 1998) and were found significantly higher in patients treated with the less compatible membranes (Kletzmayr *et al.,* 1999). An inverse correlation between IL-6 and Hb was found also in CKD patients not on dialysis (De Lima *et al.,* 2012). IL-6 has been shown also as a strong and independent predictor of ERI in 754 HD patients (Rattanasompattikul *et al.*, 2013). Some studies showed that pentoxifylline can decrease IL-6 levels (Ferrari *et al.*, 2010; González-Espinoza *et al.,* 2012). However, this not necessarily goes together with an improvement in anemia (González-Espinoza *et al.,* 2012). Beyond IL-6, IL-1, TNF-α, and IFN-γ have been found related to ESA response (Goicoechea *et al.*, 1998), especially in ESA resistant patients (Cooper *et al.,* 2003; Shooley *et al.,* 1987).

Given that pro-inflammatory cytokines also worsen oxidative stress, the latter may have an independent negative role on anemia and ESA responsiveness. On the other hand, anemia itself can worsen oxidative stress, creating a vicious circle. 8-OHdG is one of the most abundant oxidative products of DNA and it has been found increased in patients on maintenance HD (Kato *et al.,* 2003; Satoh *et al.,* 2001). Some years ago Kato *et al.* (2003) studied the association between 8-OHdG and anemia in 73 HD patients. Serum 8-OHdG had a significant inverse correlation with Hb and was also positively associated with ESA dosage and ERI. Of note, as expected the logarithm of CRP correlated inversely with Hb and positively with ESA dosage, but this inflammation index was not significantly related to 8-OHdG levels.

HD patients have higher circulating levels of MDA (Kaysen *et al.,* 2000), suggesting an accelerated lipid peroxidation. Higher MDA levels in plasma and red blood cells were negatively associated with Hb levels (Sommerburg *et al.,* 1998). Higher levels of MDA and 4-hydroxylnonenal were also found in a cohort of dialysis patients with Hb <10 g/dl compared to less- or non-anemic HD patients treated with ESA (Siems *et al.,* 2002). During HD, the concentrations of these two products of lipid peroxidation decreased (Siems *et al.,* 2002).

Carbonyl stress, which is induced by RCS, is increased in CKD patients. RCS are more stable than free radicals. For this reason, they can easily diffuse and covalently modify targets far from the site of formation. RCS are not only cytotoxic per se, but they also mediate and propagate the oxidative stress and tissue damage, acting as 'second cytotoxic messengers'. In a prospective cohort study of incident HD patients, Kalim *et al.* (2013) found that the highest quartile of carbamylated albumin was associated with a 72% higher ERI compared with the lowest carbamylation quartile. One possible pathogenetic mechanism may be a reduced erythropoietic activity of EPO following carbamylation (Mun and Golper, 2000).

Altogether, these data suggest that an efficient and biocompatible HD may be able to remove more uremic toxins (especially of higher molecular weight), decrease inflammation and thus improve anemia and response to ESA. This could be particularly true when using high flux membranes.

6.3 The role of convective treatments on anemia management

In the last decades it has been shown that dialysis dose *per se* has a significant effect on anemia (Ifudu *et al.,* 1996; Madore *et al.,* 1997). However, the relationship is lost at higher values of Kt/V (index of the dialysis efficiency) (Gaweda *et al.,* 2010; Movilli *et al.,* 2003).

Convective treatments combine more permeable and compatible membranes together with high trans-membrane flux, possibly removing medium-large molecular weight inhibitors of erythropoiesis. Accordingly, small, uncontrolled studies found anemia improvement when using high permeable and biocompatible membranes (Kawano *et al.*, 1994; Kobayashi *et al.*, 1993; Li *et al.*, 2013; Villaverde *et al.*, 1999) or high-fluxes (Ayli *et al.*, 2004). In line with this preliminary observation, a secondary analysis of the Italian Cooperative Dialysis Study showed a significant

increase in hematocrit levels in patients on high-flux compared with those on low-flux treatments (Locatelli *et al.*, 1996). More recently, data from the Japan Dialysis Outcomes and Practice Patterns Study (DOPPS) did not show any relationship between Hb levels or ESA doses and membrane biocompatibility (unmodified cellulose or biocompatible) or flux (standard or high performance) (Yokoyama *et al.*, 2008). Similarly, unpublished data of the Membrane Permeability Outcome (MPO) Study (Locatelli *et al.*, 2009) suggest no effect of high-flux dialysis on anemia.

Compared to standard convective treatments, OL-HDF combines a higher clearance of small, middle and large solutes with a lower microbiological and pyrogenic contamination of the dialysate. This may be the premise for a more significant improvement of anemia. Unfortunately data in the literature are conflicting. As it is often the case for many areas in medicine, some small uncontrolled studies gave encouraging findings (Bonforte *et al.*, 2002; Lin *et al.*, 2002; Vaslaki *et al.*, 2006). However, in addition to the small sample size, some of them had several limitations or confounding factors, such as the lack of a control group (Bonforte *et al.*, 2002; Lin *et al.*, 2002) and a concomitant increase in Kt/V (Lin *et al.*, 2002). Other small studies did not show any effect of OL-HDF on anemia correction (Ward *et al.*, 2000; Wizeman *et al.,* 2000). However, given their relative small sample size, they may have inadequate statistical power to test a difference between two efficient HD techniques (the control group received either high-flux HD (Ward *et al.*, 2000) or low-flux HD with ultrapure dialysate and biocompatible membrane (Wizeman *et al.,* 2000)).

A meta-analysis of 65 studies (12,182 patients) compared all convective therapies (including high-flux HD, hemofiltration and HDF) vs. low-flux HD on several outcomes (Susantitaphong *et al.,* 2013). In the study arms receiving convective treatment, a trend towards a decrease in ERI (-2.51 U/week/kg/g/dl; confidence interval: -5.24 to 0.21; P=0.07) was observed compared to low-flux HD. Of note, information about anemia was available only in 25 studies (n=1504), which had significant heterogeneity.

In the last few years, several trials tested the role of either pre-dilution or post-dilution OL-HDF and HF on hard or intermediate end-points. The majority of them did not find a significant effect of OL-HDF or HF on anemia parameters and ESA doses in comparison to standard HD treatments (Grooteman *et al.,* 2013; Locatelli *et al.*, 2012; Maduell *et al.,* 2013). The Turkish Online Haemodiafiltration Study (Ok *et al.*, 2013), which randomized 782 HD patients to either post-dilution online HDF (filtration volume of 17.2±1.3 l) or high-flux HD, was the only one who found a lower mean ESA dose in the HDF group compared to the HD one.

Starting from the hypothesis that high-convection volumes are needed to improve survival (Maduell *et al.*, 2013), the REDERT study was designed to test the effect of high-volume (>20 l/session) HDF on ERI and hepcidin levels (Panichi *et al.,* 2015). In this cross-over study, 40 HD patients were randomized to either online HDF or standard bicarbonate HD. During the HD period higher ESA doses were administered compared to the HDF period (192,444±131,341 vs. 135,955±96,070 UI/six months, respectively; P<0.001). Hepcidin levels were also reduced in HDF compared to standard HD. This preliminary data suggests to further explore the hypothesis of a dose effect of OL-HDF on anemia with higher convective volumes.

Several factors should be taken into account as an explanation for the different results of the various trials. First, the majority of the trials enrolled patients with a low comorbidity burden, possibly reducing the chance to observe a significant effect of HDF on anemia (as it was the case for dialysis dose per se). Second, the improved anemia in patients treated with HDF may be due to the use of ultra-pure dialysate. In this respect, some studies that found a beneficial effect did not use ultra-pure dialysis fluid in the control group.

6.4 Vitamin E coated membranes

Alfa-tocopherol, which is one of the eight isoforms of vitamin E, is the most potent, fat-soluble antioxidant known in nature. It inhibits LDL oxidation and protects cells from oxidized LDL. It also reduces the levels of LDL(-), a particle possibly involved in atherogenesis, which is present at high concentrations in HD patients (Mafra *et al.,* 2009). Vitamin E-coated membranes have been developed (Piroddi *et al.*, 2012) to decrease oxidative stress in HD patients and improve outcome.

In CKD patients several clinical trials tested the efficacy of vitamin E on anemia either using the oral administration route or taking advantage of the dialysis procedure by means of vitamin-E-containing dialyzers (Table 6.1).

Early in 2002, Usberti *et al.* studied the combined effect of oral antioxidant and a low-biocompatible vitamin-E coated membrane in 38 HD patients. Despite a positive effect on anemia, according to the study design, it was not possible to evaluate the contribution of the single treatments. Some years later, Cruz *et al.* (2008) were the first to formally test the role of vitamin E coated membrane on anemia. They shifted 172 HD patients from their previous dialyzer to a vitamin-E coated membrane and followed them for 12 months. A significant improvement in the percentage of patients achieving adequate anemia control was found (from 49.4% at baseline to 80% at study end, together with a lower ESA dose (from 7,762±5,865 IU/week to 6,390±5,679 IU/week). However, the possible efficacy of vitamin E was confounded by the lack of a control group and by the fact that the new dialyzer had enhanced membrane biocompatibility.

To circumvent these limitations, Andrulli *et al.* (2010) performed a pilot randomized, parallel-group study of 20 HD patients, who were assigned to high-flux synthetic polysulfone dialyzer with or without vitamin E. During the eight-month follow-up, ERI decreased more in the vitamin-E treated group (-37%) than in the control group (-20%), but this difference was not statistically significant (P=0.596). In a multivariate analysis, after adjusting for baseline values of parathyroid hormone (PTH), γ and α-tocopherol, the vitamin-E dialyzer effect on ERI became statistically significant (P=0.042). Another small, pilot, cross-over study, who selected only HD patients with central vein catheter, compared a vitamin E-bonded dialyzer with a polyethersulfone membrane over six months of follow up in 16 HD patients (Mandolfo *et al.,* 2012). In this selected HD population, which is usually affected by a high burden of comorbidities, ERI decreased more with the vitamin-E dialyzer compared to that without (8.7±5.2 and 12.1±5.2, respectively; P<0.0001). A trend towards changes in protein glycoxidation, antioxidant, and inflammatory markers was

Table 6.1. Studies testing the effect of vitamin E coated membranes on anemia in hemodialysis patients.

Study	Design	N	Treatment	Control group/ treatment at baseline	Effect on anemia/ erythropoietin stimulating agent[1]	Follow up
Usberti *et al.* (2002)	observational, prospective	38	low-biocompatible vitamin E membrane + oral antioxidant	no control group	Hb ↑ ; ESA ↓	not available
Cruz *et al.* (2008)	observational, prospective	172	high biocompatible vitamin E membrane	no control group	↑ % of patients with adequate anemia control; ESA ↓	12 months
Andrulli *et al.* (2010)	controlled, randomized	20	high-flux synthetic polysulfone dialyzer with vitamin E	high-flux synthetic polysulfone dialyzer without vitamin E	ERI ↓ at multivariate analysis	8 months
Panichi *et al.* (2011)	two-arm, crossover	62	high-flux, vitamin E-coated polysulfone membrane	high-flux polysulfone membrane	Hb ↑ and ERI ↓ in the vitamin E group	each arm, 6 months
Mandolfo *et al.* (2012)	two-arm, crossover	16 (with central vein catheter)	high-flux polyethersulfone membrane with vitamin E	high-flux polyethersulfone membrane without vitamin E	ERI ↓	each arm, 6 months
Sanaka *et al.* (2013)	controlled, randomized	305	high-flux vitamin E-coated polysulfone membrane	high-flux polysulfone membrane	overall ERI =; ERI ↓ in the Hb group 11-11.9 g/dl	12 months
Lines *et al.* (2014)	controlled, randomized	260	high-flux vitamin E-coated polysulfone membrane	high-flux polysulfone membrane	overall ERI =; ERI ↓ in the highest ERI tertile at baseline	12 months
Locatelli *et al.* (unpublished data)	controlled, randomized	94	high-flux vitamin E-coated polysulfone membrane	low-flux dialyzer	ERI ↓ in the vitamin E group and ERI ↑ in the control group	12 months

[1] ERI: erythropoiesis resistance index; ESA: erythropoietin stimulating agent; Hb: hemoglobin; ↑ : increase; ↓ :decrease; =:no effect.

also observed. It is possible that in this study the higher efficacy of vitamin-E coated membrane on anemia parameters may be due to the fact that patients with central vein catheter are more likely to be inflamed and ESA hyporesponsive and thus have higher room for improvement.

In 2011 Panichi *et al.* performed a randomized study including 62 HD patients, who had been receiving standard bicarbonate HD with a synthetic membrane in the previous month and who were shifted to either polysulfone + vitamin E or to polysulfone dialyzer alone for six months

in a cross-over design. While Hb remained unchanged with polysulfone HD, it significantly increased with the adding of vitamin E in the dialyzer (from 11.1±0.6 g/dl at baseline to 11.5±0.7 at six months). In parallel, ERI significantly decreased only in the vitamin-E period (from 10.3±2.2 IU dl/kg/g Hb week at baseline to 9.2±1.7 at six months). Interestingly, these changes in anemia parameters went together with a significant decrease in IL-6 levels.

More recently, data from two larger randomized clinical trials have somehow questioned the efficacy of vitamin E-bonded membranes on anemia parameters. A multicenter, Japanese, prospective study enrolled 305 HD patients and randomized them to four treatment groups according to vitamin E (yes/no) polysulfone dialyzer and to two Hb target ranges (from 10.0 to 10.9 g/dl and from 11 to 11.9 g/dl depending on run-in Hb levels) (Sanaka *et al.,* 2013). After one year, overall no significant difference in ERI was found between the two high-flux HD treatments (one with and one without vitamin E). Weirdly, the separate analysis of the two Hb target ranges showed a reduction in ERI together with a lower variability with the vitamin E dialyzer only in the Hb range of 11-11.9 g/dl. Considering the lower ESA doses of the Japanese HD population in comparison to Western countries, this secondary analysis may suffer of inadequate statistical power. Indeed, median recombinant human erythropoietin doses were of only 4,500 and 3,000 U/week in the lower and higher Hb target groups, respectively. The lower ESA dose needs in the higher Hb target group may also suggest the random selection of patients with a lower burden of comorbidities in this subgroup, further complicating the interpretation of the findings. Unfortunately, no assessment of inflammation markers was performed. The study had also several drawbacks, such as a high number of dropouts (92 out of 305 patients; 30%), a very low number of analyzed patients per center in relation with the number of treatment groups (4.4 patients per center on average with four treatment groups, that is equivalent to 1.1 patients/treatment group), the lack of multivariate approach and the inflation derived from the use of multiple statistical tests. Finally, differing from other studies (Andrulli *et al.,* 2010), ERI increased up to 40% in the control group during follow-up.

Lines *et al.* (2014) performed another large randomized clinical trial in the United Kingdom enrolling more than 200 HD patients. Differing from other studies, all ESA dosing was performed by means of a computer-based decision support system. Compared to polysulfone alone, overall no significant effect of the vitamin E-bonded membrane was found on ERI after 12 months (P=0.08). Of note, at baseline the patients in the vitamin E group were diabetics in a higher percentage and had a higher median unadjusted ESA dose, possibly reducing the experimental treatment effect. After stratifying the patients in tertiles of baseline ERI, a significant reduction was found only in those patients with the highest baseline ESA resistance dialyzing with the vitamin E membrane. No correlation was found between the change in ERI and CRP, despite significantly higher CRP values at baseline in this tertile. However, the CRP values have been found an insensitive parameter in this respect (Panichi *et al.*, 2008).

Finally, very recently a meta-analysis of 14 trials (974 HD patients) on several outcomes has been published (Huang *et al.,* 2015). Data on anemia were available only in six trials; only three gave information on ERI with acceptable heterogeneity. Vitamin E-coated dialyzer could decrease ERI

(standardized mean difference: -0.24; 95% confidence interval: -0.47 to -0.01; *P*=0.04). However, strangely enough the separate analysis of ESA doses and Hb levels did not show any significant effect (of note, heterogeneity among studies was significant).

Recently our group coordinated an Italian, controlled, multicentre, randomized study (Locatelli *et al.*, unpublished data). 94 HD patients on stable ESA therapy were randomized to either a vitamin E-coated polysulfone dialyser (experimental treatment) or to a low-flux dialyzer (control treatment). The majority of the patients (93%) were followed for at least four months; 57 patients (61%) completed the planned 12-month follow-up. Using the last available data approach, ESA resistance decreased in the vitamin-E group of 1.06±1.35 IU/(kg over g/dl) and increased in the control group of 1.21±1.34 IU/(kg over g/dl) with a significant mean end-follow-up difference of 2.27 IU/(kg over g/dl) (*P*=0.041). According to these findings, vitamin E placed on the blood-side of a dialyzer may have a beneficial effect on ESA resistance in HD patients.

6.5 Conclusions

Altogether, the theoretical possibility that highly-efficient and biocompatible HD may improve anemia is intriguing. Vitamin E coated dialyzer would add a selective benefit on oxidative stress. Unfortunately, data from available studies are conflicting, with larger trials giving negative findings. The reason of smaller positive effects compared to expectations may have several reasons. First, many studies compared these new HD techniques with already efficient HD, thus reducing the difference between the two treatments. Second, it is possible, as suggested by the positive findings with vitamin E coated membranes in ESA hyporesponsive patients, that the effect of convective treatments and/or vitamin E coated membranes is mainly due by a general decrease in the inflammatory status of the patients, making more inflamed patients more sensitive to the treatment effect.

Overall, convective treatment and vitamin E-coated dialyzers seem to have a quite small effect on anemia parameters in the dialysis population as a whole, provided that HD is adequate and the quality of water is high. Conversely, they seem to have some positive effect in inflamed patients.

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7. Conjoint deficiency of vitamin C and NQO1 causes cigarette smoke-induced myelodysplastic syndrome

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Abstract

Myelodysplastic syndrome (MDS) is a type of cancer that shares many characteristics with acute myelogenous leukemia. Till today, the cause of MDS is poorly understood. Risk of MDS is associated with cigarette smoke exposure. The link between increased NAD(P)H: quinone oxidoreductase 1 (NQO1) deficiency and risk of MDS is inconsistent. Also, not much is known about the influence of vitamin C in MDS development. It is very complicated to understand the relationship between cigarette smoke exposure, NQO1 deficiency and vitamin C in population-based studies. We addressed this relationship in a guinea pig model. Guinea pigs having conjoint deficiency of NQO1 (fed 3 mg dicoumarol/d) and marginal vitamin C deficiency (fed 0.5 mg vitamin C/d) were exposed to cigarette smoke for 21 days. These guinea pigs developed MDS. We observed that p-benzosemiquinone derived from cigarette smoke is a causative factor of MDS. MDS was characterized by refractory cytopenia, multilineage dysplasia with myeloid hyperplasia, aneuploidy and increased CD34+ cells. The initial events were oxidative damage and apoptosis. Oxidative damage was indicated by the formation of protein carbonyls and 8-oxodeoxyguanosine. Apoptosis was evidenced by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, increase in the levels of phosphorylated p53 and cleaved caspase3 as well as increased ratio of Bax/Bcl-2. MDS appeared after disappearance of apoptosis. Prior administration of vitamin C (30 mg vitamin C/kg body weight/d), an antagonist of p-benzosemiquinone, prevented MDS. In totality, our results suggest that human smokers having conjoint deficiency of NQO1 and marginal vitamin C would be at high risk for developing MDS, and regular intake of vitamin C (approximately 2 g/adult human/d) should prevent occurrence of cigarette smoke-induced MDS.

Keywords: guinea pig, p-benzoquinone, cytopenia, dysplasia

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Key facts

- A myelodysplastic syndrome (MDS) is a type of cancer. Approximately one-third of MDS patients ultimately progress to acute myelogenous leukemia. MDS patients have a poor survival.
- MDS is primarily a disease of the elderly. The median age at diagnosis of a MDS is between 60 and 75 years. Death from MDS is often caused by bleeding and infection.
- Prevalence of MDS is 50,000-100,000 cases in the USA. About 15,000-30,000 new cases of MDS are diagnosed each year.
- MDS is not generally inherited. It occurs after long-term exposure to benzene or cigarette smoke (*de novo* MDS) or after receiving chemotherapy or radiation therapy (therapy related MDS).
- Treatment of MDS involves supporting care and the use of agents aimed at restoring normal hematopoiesis. However, allogenic stem cell transplantation represents the only potentially curative treatment.

Summary points

- MDS is a type of elderly cancer that shares many characteristics with acute myelogenous leukemia. MDS patients have poor survival.
- The etiology of MDS is poorly understood. We have produced MDS within 21 days by exposure of cigarette smoke to marginal vitamin C-deficient guinea pigs having conjoint deficiency of NAD(P)H: quinone oxidoreductase 1 (NQO1).
- The MDS produced in the guinea pigs falls in the category of refractory cytopenia with unilineage dysplasia: refractory anemia; refractory thrombocytopenia with myeloid hyperplasia and aneuploidy.
- The risk factor in cigarette smoke for causing MDS is p-benzosemiquinone, which is produced in the smoker lungs, goes to the bloodstream and the bone marrow, the target organ.
- Although the molecular mechanism of cigarette smoke-related MDS is not clear, it appears to be p-benzosemiquinone-induced apoptosis, arylation and oxidative damage of proteins and DNA.
- Prior feeding of vitamin C at a dose of approximately 30 mg/kg body weight prevents cigarette smoke-induced MDS. However, vitamin C is ineffective after the onset of MDS.
- Results obtained with guinea pigs would indicate that regular intake of vitamin C (approximately equivalent to 2 g/adult human/d) should prevent occurrence of MDS in chronic smokers.

Abbreviations

8-oxodG	8-oxo-7,8-dihydroguanosine
AML	Acute myelogenous leukemia
BP	Benzo[a]pyrene
CS	Cigarette smoke
DAPI	4, 6-diamidino-2-phenylindole
DC	Dicoumarol (3,3'-methylene-bis(4-hydroxycoumarine))
EGFR	Epidermal growth factor receptor
MDS	Myelodysplastic syndrome
NNK	4-(methylnitrosamino)-1-(3-pyridil)-1-butanone
NQO1	NAD(P)H: quinone oxidoreductase 1
p-BQ	p-benzoquinone
p-BSQ	p-benzosemiquinone
PI	Propidium iodide
ROS	Reactive oxygen species
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

7.1 Introduction

MDS is a type of cancer that shares many characteristics with AML. The bone marrow shows hypercellularity, arrested maturity of one or more cellular lineages, and an increase in bone marrow myeloid precursors (Attar, 2008). In majority of cases, the disease is chronic with gradually worsening cytopenia due to progressive bone marrow failure. MDS patients have a poor survival. Cytopenias, a hallmark feature of MDS, are responsible for some of the symptoms that MDS patients experience – infection, anaemia, spontaneous bleeding, or easy bruising. MDS is primarily a disease of the elderly. The median age at diagnosis of a MDS is between 60 and 75 years.

Until now, treatment options for MDS patients include mainly supportive care, chemotherapy and allogenic stem cell transplantation (Anargyrou *et al.*, 2010). But these procedures have had little impact on MDS survival in general. There has been little progress toward the identification of new markers and therapeutic targets of MDS, particularly because the exact cause of MDS is unknown in most patients. The genetic factor(s) involved in increased susceptibility to MDS are not known. MDS may be *de novo* and therapy related. Radiation and/or chemotherapy for cancer are among the known triggers for the development of therapy related MDS in a portion of patients (Park and Koef er, 1996; Strom *et al.*, 2008). *De novo* MDS develop after long term exposure to certain environmental or industrial chemicals, such as benzene (Nebert, 2002; Smith, 1996) and CS (Björk *et al.*, 2000, 2009; Du *et al.*, 2010; Lichtman, 2007; Pasqualetti *et al.*, 1997; Strom *et al.*, 2008; Tong *et al.*, 2013). In the case of benzene-induced MDS, the risk factor may be attributed to its metabolite p-BQ, which targets to the bone marrow (Nebert, 2002; Smith, 1996).

We have also observed that the risk factor derived from CS causing MDS attributes to p-BQ (Das *et al.*, 2011). In smokers' lung, p-BQ is produced from p-BSQ, which is substantially present in all commercial cigarettes (100-200 μg/cigarette). p-BSQ is converted to p-BQ through disproportionation (Sullivan and Reynolds, 1976) and oxidation by transition metal containing proteins (Banerjee *et al.*, 2008; Chatterjee, 2004; Dey *et al.*, 2010; Ghosh *et al.*, 2015). p-BQ reaches the bone marrow through blood stream and targets ε-amino groups of lysine residues of proteins to form covalent adducts and thereby exerts its effect accordingly (Das *et al.*, 2011).

NQO1, an enzyme present in bone marrow including other tissues (Bauer *et al.*, 2003; Nebert, 2002; Ross, 2005; Smith, 1999) detoxifies p-BQ. p-BQ-induced toxicity of the hematopoietic system would be protected by normal NQO1 activity. On the other hand, the risk of bone marrow toxicity would be increased in NQO1 deficient state. However, the results in different populations are inconsistent. A few reports show that lack of NQO1 activity does not correlate with increased risk of malignancy (Lan *et al.*, 2004; Steiner *et al.*, 1999; Yin *et al.*, 2001).

Besides enzymatic reduction by NQO1, vitamin C also reduces and inactivates p-BQ to a great extent. However, the role of vitamin C on MDS is yet to be known. We previously showed that marginal vitamin C-deficiency was associated with CS-induced toxicity and tissue damage in guinea pigs, and the damage was prevented by a moderately large dose of vitamin C (Banerjee *et al.*, 2008; Panda *et al.*, 1999, 2000). Thus we considered that nutritional status of vitamin C in addition to NQO1 deficiency might be a critical determining factor for MDS.

The role of vitamin C in the prevention and treatment of cancer has been indicated by lots of epidemiological studies, including leukemia. However, this has been a subject of debate for a long time. (Head, 1998; Lee *et al.*, 2003). In leukemic patients, plasma vitamin C levels were practically undetectable (Waldo and Zipf, 1955). Growth and weight of human, rat and murine tumor xenografts in athmyc, nude mice are decreased by pharmacological doses of vitamin C (Frei and Lawson, 2008). A series of case reports indicated that high-dose intravenous vitamin C was associated with long-term tumor regression in patients with advanced renal cell carcinoma, bladder carcinoma, or B-cell lymphoma (Padayatty *et al.*, 2006). This would indicate that high doses of vitamin C are safe and well tolerated in different groups of patients (Frei and Lawson, 2008).

Population-based study is troublesome to determine the relationship among CS exposure, NQO1 deficiency and marginal vitamin C deficiency. We have used a guinea pig model to address this issue and here we show that CS exposure causes MDS in guinea pigs having marginal vitamin C deficiency conjoint with NQO1 deficiency. CS-induced MDS is not caused by any singular deficiency of vitamin C or NQO1. Like humans, guinea pigs cannot synthesize vitamin C and they are solely depended on the dietary source (Chatterjee, 1973). Also, the guinea pig has anatomical and CS-induced pathophysiological similarities to humans (Wright and Churg, 2002). We therefore used guinea pig as a model animal in our study.

7.2 Vitamin C status and NQO1 activity level in bone marrow of guinea pigs after conjoint deficiency

English male short hair guinea pigs (350-450 g body weight) were fed 0.5 mg vitamin C/d and 15 mg vitamin C/d, respectively, to produce marginal vitamin C-deficient and vitamin C-sufficient guinea pigs respectively (Das *et al.*, 2011, 2012). Using HPLC, we measured the vitamin C level in bone marrow lysate to which β-mercaptoethanol (0.13 mM) was added to prevent oxidation of the vitamin (Banerjee *et al.*, 2008). After feeding vitamin C-deficient diet at a dose of 0.5 mg/d for 21 days along with CS exposure, the vitamin C level in bone marrow is not detectable as shown in Table 7.1. DC, a potent inhibitor of NQO1 (Tsvetkov *et al.*, 2005) was fed at a dose of 3 mg/d in aqueous suspension for 21 days to make NQO1 deficiency. As shown in Figure 7.1, DC-treated group produced 90% inhibition of NQO1 activity compared to control (Air). DC competes with

Table 7.1. Vitamin C levels in the bone marrow of guinea pigs exposed to air or CS for 21 days.[1]

	Vitamin C supplementation (mg/d)	
Group	0.5	15
Air	0.18±0.02	5.17±0.29
Dicoumarol	0.13±0.01	2.27±0.06
Cigarette smoke	ND	2.65±0.20
Dicoumarol + cigarette smoke	ND	2.75±0.10

[1] The vitamin C contents of bone marrow were estimated on the 21st day by HPLC as described by Das *et al.* (2011). ND: not detectable. Data represent mean ± standard deviation (n=4) (reproduced from Das *et al.*, 2011).

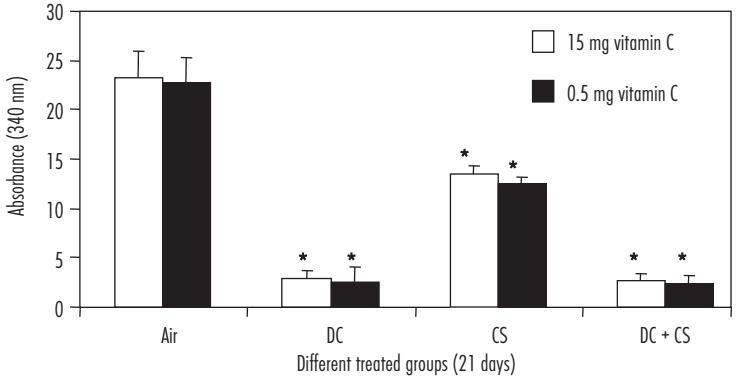


Figure 7.1. NQO1 activity of bone marrow cells of guinea pigs fed 0.5 mg or 15 mg vitamin C/d. Air: exposed to air; CS: exposed to cigarette smoke; DC: fed 3 mg dicoumarol/d; DC+CS: fed 3 mg dicoumarol/d and exposed to cigarette smoke. *: significantly different ($P<0.05$) with respect to air exposed guinea pigs. Bars over the respective columns represent means ± standard deviation (n=6) (reproduced from Das *et al.*, 2011).

NAD(P)H for binding to NQO1 and thereby inhibits NQO1 activity (Asher, 2006). The guinea pigs were exposed to CS from five Kentucky Reference cigarettes: 3R4F (2 puffs/cigarette) per animal/d (6 days/week) in a smoke chamber (Banerjee *et al.*, 2008) with a duration of 90 seconds/puff. Pair-fed sham controls were exposed to air in place of CS under same conditions. After the experimental period, the animals were euthanized using intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight). All methods were approved by the Institutional Animal Ethics Committee, permit No. 797/CPCSEA.

7.3 Morphological, histological and cytogenetic evidence of cigarette smoke-induced myelodysplastic syndrome

According to WHO classification (Vardiman *et al.*, 2009) based on bone marrow cell morphology, histology and cytogenetic evidence, the MDS diagnosed in guinea falls in the category of refractory cytopenia with unilineage dysplasia: refractory anemia; refractory thrombocytopenia. Irreversible MDS occurred in the guinea pigs having conjoint deficiency of NQO1 and vitamin C after exposure to CS for 21 days, but not in vitamin C-sufficient ones. After the onset of MDS, administration of vitamin C (15 mg/d) did not attenuate the pathophysiological conditions (Das *et al.*, 2011).

Leishman staining showed some typical changes in blood cell morphology (Figure 7.2). Figure 7.2B shows hyper segmented neutrophils in the blood film indicating dysgranulopoiesis, compared to

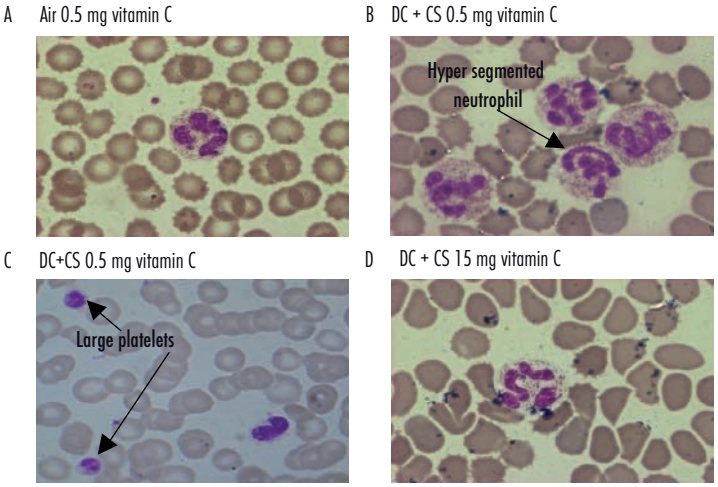


Figure 7.2. Identification of myelodysplastic syndrome in guinea pigs by blood cell morphology. Differential staining showing some typical changes in blood cell morphology of myelodysplastic syndrome guinea pigs and prevention by feeding the animals 15 mg vitamin C/d. Leishman stain, magnification 1000× (reproduced from Das *et al.*, 2011). CS: cigarette smoke; DC: dicoumarol.

normal segmented neutrophils. In comparison to blood platelets of guinea pigs exposed to air and fed 0.5 mg vitamin C/d (Figure 7.2A), MDS guinea pigs depicts poorly functioning large platelets (Figure 7.2C). There was remarkable decrease in hemoglobin and red blood cell parameter but white blood cell count was unchanged. These pathological changes were not found in vitamin C-sufficient (fed 15 mg vitamin C/d) ones (Figure 7.2D).

Bone marrow smears stained separately with Wright-Giemsa, myeloperoxidase, Sudan Black, and Perls' stain (Lewis and Bain, 2006) indicated that bone marrow dysplasia was present in all three lineages with predominance in erythroid (15%) and granuloid (9.5%) series. In megakaryocytes, dysplastic cells were 8% with <5% (4.88±0.63%) blast cells. Also, less than 15% erythroid precursors were ring sideroblast (6%). Because all three lineages were affected, the disorders probably occurred at the pluripotent stem cells.

In erythroid lineage, the guinea pigs fed 0.5 mg vitamin C and exposed to air shows normal megaloblast, a large cell with dark blue cytoplasm and primitive nuclear chromatin pattern (Figure 7.3AI). Contrary to this, Figure 7.3AIV shows several changes like marked hypercellular vacuolation with some megaloblastoid changes. Figure 7.3AVII shows ring sideroblasts as evidenced by many blue siderotic rings surrounding the nucleus. This indicates dysethropoiesis (CantùRajnoldi *et al.*, 2005). Perls' stain positive bone marrow (Figure7.3AX) showing iron accumulation in erythroblastsis is another evidence of dyserythropoiesis (Platzbecker *et al.*, 2007; Seo *et al.*, 1993). This is in contrast to the group fed 0.5 mg vitamin C and exposed to air, which shows normal megaloblast with dark blue cytoplasm and primitive nuclear chromatin (Figure 7.3AI). The dyserythropoiesis was prevented by feeding the animals 15 mg vitamin C/d (Figure 7.3AXIII)

In granuloid lineage, in contrast to normal myelocyte (Figure 7.3II), MDS guinea pigs show dysgranulopoiesis as evidenced by dysplastic myelocyte with abnormal separation of nuclear lobes (Figure 7.3AV) and atypical mononuclear cells with altered nuclear and cytoplasm ratio (Figure 7.3AVIII). Moreover, MDS group shows considerable increase of myeloblast (4.88+0.63%), the first recognizable cell in granuloid series (Figure 7.3AXI). Dysgranulopoiesis was prevented by feeding 15 mg vitamin C/d (Figure 7.3AXIV).

In megakaryocytic lineage, bone marrow megakaryocytes population in MDS guinea pigs increased significantly (P<0.05, n=6), but the total platelet count decreased (Das *et al.*, 2011). This indicates occurrence of dysplastic megakaryocytes or micromagakaryocytes (Figure 7.3A, VI, IX and XII). This evidences dysmegakaryopoiesis (CantùRajnoldi *et al.*, 2005). Figure 7.3A, III and XV show normal megakaryocytes. As reported by others (Platzbecker *et al.*, 2007; Reis *et al.*, 2009), we also observed that compared to control and other experimental groups (Figure 7.3B and C), CD 34(+) cells increased significantly (about 10%, P<0.05, n=6) in bone marrow of MDS guinea pigs.

Aneuploidy, numerical chromosomal aberrations, is a common feature in cancer including hematopoietic tumor cells (Ganmore *et al.*, 2009). The guinea pig has 62 chromosomes (Gava

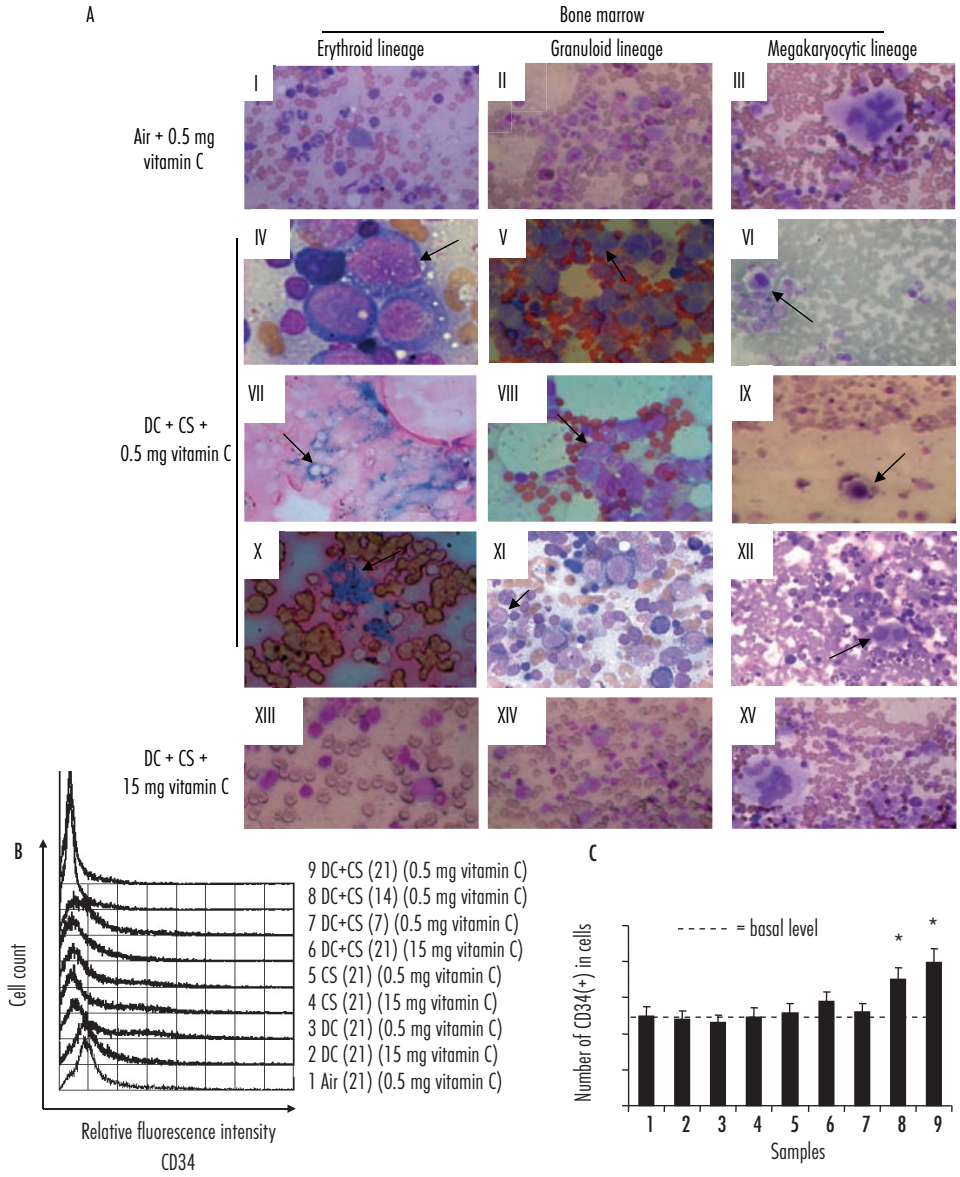


Figure 7.3. Identification of myelodysplastic syndrome (MDS) in guinea pigs by morphological changes in bone marrow cells including measurement of CD34(+) cells. (A) Differential staining showing some typical changes in bone marrow cell morphology of MDS guinea pigs and its prevention by feeding the animals 15 mg vitamin C/d. (AI-III) Represent sham controls (fed 0.5 mg vitamin C/d and exposed to air); (AIV-XII) represent MDS guinea pigs (cigarette smoke (CS)-exposed fed dicoumarol (DC) and 0.5 mg vitamin C/d); (AXIII-XV) represent CS-exposed guinea pigs fed DC and 15 mg vitamin C/d. Wright Giemsa stain; AVII and AX: Perls' stain (Lewis and Bain, 2006). Magnification 400×; AIV: 1000×; → indicates dysplastic cells. (B) Measurement of CD34(+) cells in bone marrow by flow cytometry. (C) Quantitative evaluation of CD34(+) cells. Bars over the respective columns represent mean ± standard deviation (n=6); *: significantly different (P<0.05) with respect to samples 1-7 (reproduced from Das *et al.*, 2011).

et al., 1998). We found that compared to control guinea pigs (karyotype 60+XY, Figure 7.4A), the chromosome number in MDS guinea pigs varied from 116-128 (Figure 4B). Analyses of 100 metaphases showed that 30% metaphases represented aberrations. The remaining 70% was normal karyotype. This shows aneuploidy and indicates genomic instability.

Taken together, the above mentioned results indicate that CS-exposed guinea pigs having conjoint deficiency of NQO1 and vitamin C develop MDS. However, administration of 15 mg vitamin C/d prevented the onset of MDS.

7.4 Role of apoptosis in myelodysplastic syndrome

A number of reports indicate that apoptosis has a role in MDS (Blagosklonny 2000; Bogdanović *et al.*, 1997). Interestingly we found that bone marrow apoptosis is the initial event that is followed by MDS. Using TUNEL assay and flow cytometric analysis we confirmed that TUNEL (+) cells and apoptotic population (Figure 7.5C; Annexin V+/PI-) in bone marrow cells markedly increased after CS exposure for 7 and 14 days (Figure 7.5A). However, after 21 days apoptosis disappeared, but MDS appeared (Figure 7.5A and B). This suggests that apoptosis is followed by MDS. Apoptosis and MDS were absent in vitamin C-sufficient group. Flow cytometry analysis also confirmed the increase (24.52%) of apoptotic population (Annexin V+/PI-) on day 14, which was absent 0.12%) on day 21 (Figure 7.5C. There was practically very little necrosis.

Consistent with TUNEL and flow cytometric analyses, apoptosis was confirmed by marked increase of phosphorylated p53 (p-p53) level in bone marrow cells on day 14, when apoptosis was high (Figure 7.5E, row 1). p53 was absent on day 21 (Figure 7.5D and E, row 1), when MDS appeared. This was corroborated by an increase in Bax/Bcl-2 ratio and cleaved caspase 3 on day 14 (Figure 7.5E). It is reported that TNF-α is associated with apoptosis in early MDS (Platzbecker *et al.*, 2007). Here we show that on day 14, TNF-α level and its downstream effector cleaved caspase

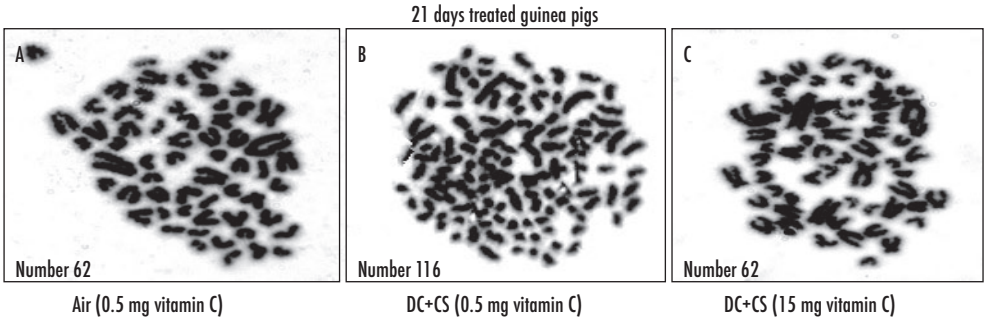


Figure 7.4. Numerical chromosomal aberration in myelodysplastic syndrome guinea pig. Giemsa-stained metaphase spread showing aneuploidy in B (magnification 1000×) (reproduced from Das *et al.*, 2011). DAPI: 4,6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelling.

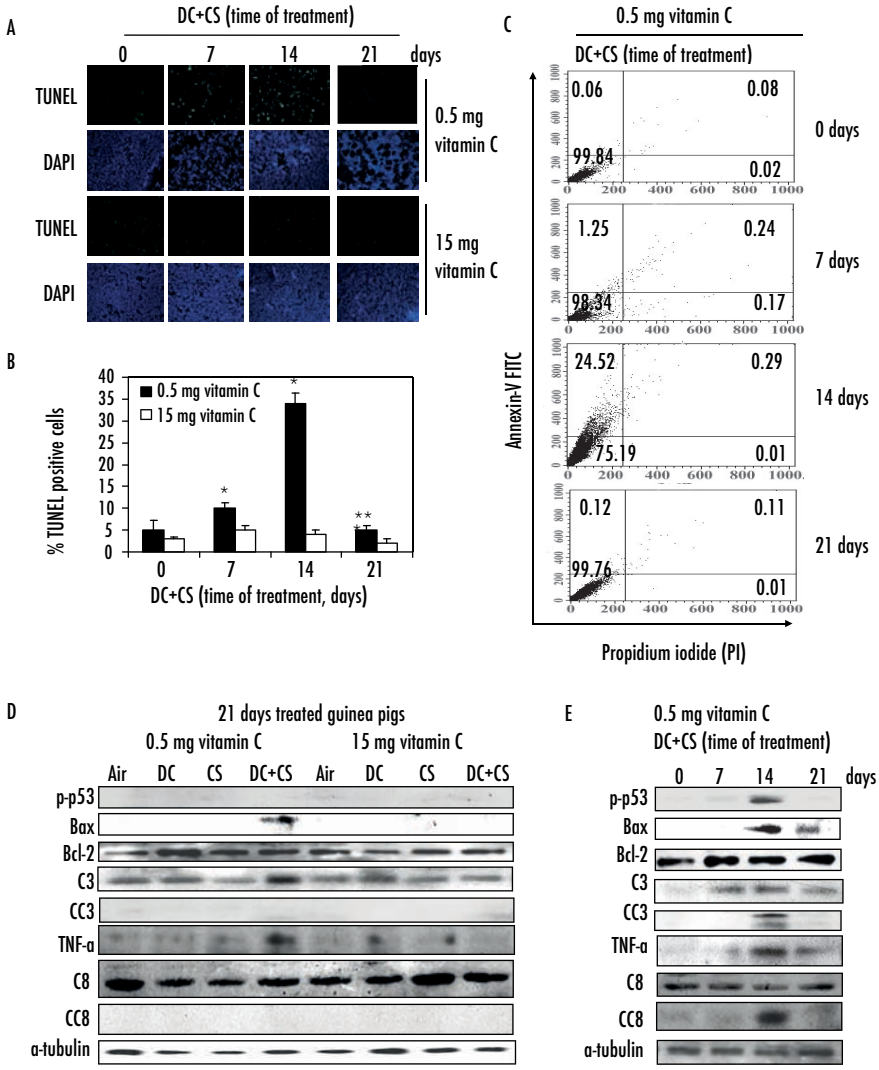


Figure 7.5. TUNEL assay, flow cytometric analysis and apoptotic signaling in bone marrow. (A) TUNEL assay of bone marrow cells; stained with fluorescein labeled dUTP and 4,6-diamidino-2-phenylindole (DAPI), respectively. Green fluorescence indicates TUNEL positive cells (magnification 200×). (B) Quantitative evaluation of TUNEL positive cells; bars over the respective columns represent TUNEL positive cells (mean ± standard deviation, n=6), *: significantly different ($P<0.05$) with respect to day 0, **: significantly different ($P<0.05$) from 14 days treatment. (C) Flow cytometry analyses of bone marrow cells using Annexin V-FITC fluorescence (y-axis) vs. propidium iodide (x-axis). Quadrants: lower left, viable cells; upper left, apoptotic cells; upper right, late apoptotic and lower right, necrotic cells. (D) and (E) Immunoblots of p-p53, Bax, Bcl-2, caspase 3 (C3), cleaved caspase 3 (CC3), TNF-a, caspase 8 (C8) and cleaved caspase 8 (CC8) (reproduced from Das *et al.*, 2011).

eight increased markedly. TNF-α decreased significantly and caspase 8 was absent on day 21 (Figure 5D and E, rows 6 and 8, respectively).

7.5 Bone marrow hypercellularity in myelodysplastic syndrome

Cell cycle analyses have demonstrated increased cellular proliferation of bone marrow cells in MDS guinea pigs, particularly in the myeloid lineage (Attar, 2008). It has been reported that in malignancies including leukemia EGFR activation is required for proliferation. Also Akt pathway plays an important role for cell survival and proliferation in high-risk MDS patients. We observed that EGFR activation occurred on day seven that continued up to day 21 (Figure 7.6A). EGFR activation was accompanied by activation of Ras, ERKI/2, p-ERKI/2, c-Myc, p-c-Myc,

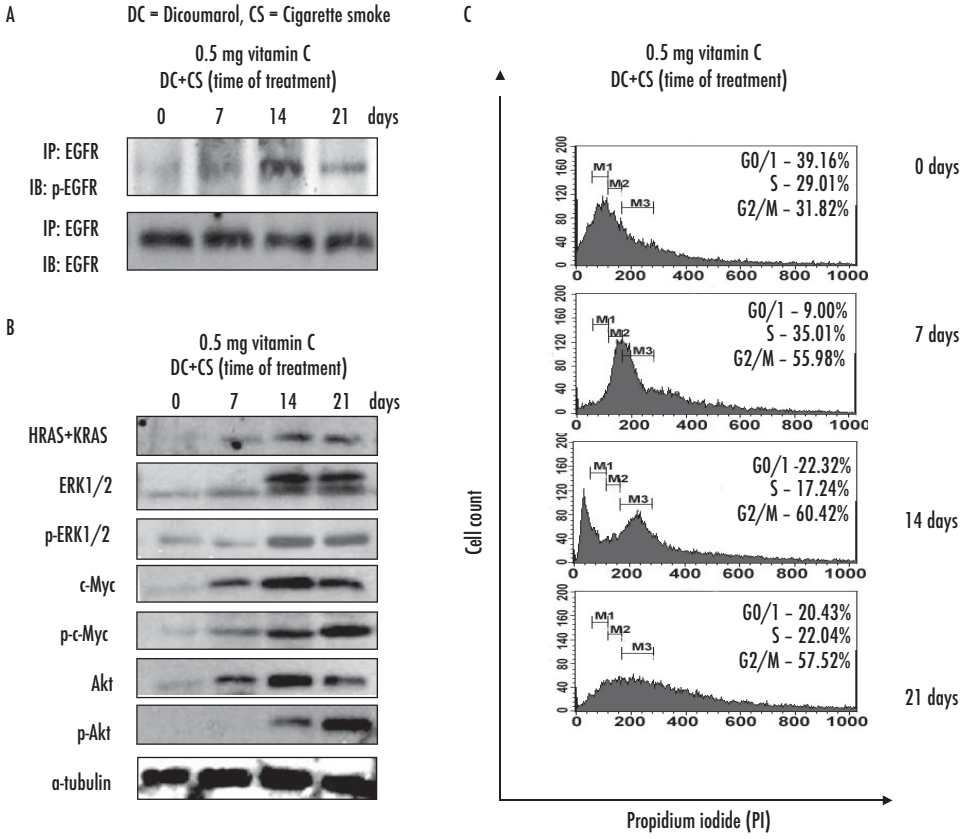


Figure 7.6. Cell cycle and signaling mechanism in myelodysplastic syndrome guinea pigs. (A) Epidermal growth factor receptor (EGFR) was immunoprecipitated (IP) with anti-EGFR and the precipitated proteins were immunoblotted (IB) with anti-phospho-Tyr (PY20) and anti-EGFR. (B) Immunoblots of HRAS+KRAS, ERK1/2, p-ERK1/2, c-Myc, p-c-Myc, Akt and p-Akt. (C) Cell cycle analyses of bone marrow by flow cytometry: y-axis, cell counts; x-axis. Propidium iodide stain (reproduced from Das *et al.*, 2011).

Akt and p-Akt (Figure 7.6B). No bone marrow hypercellularity was observed in guinea pigs fed 15 mg vitamin C/d.

Cell cycle analyses (Figure 7.6C) further demonstrated that compared to day 0, there was significant decrease in the number of G0/1 and S phase and increase in the number ($P<0.05$) of G2/M-phase cells after 21 days treatment, when MDS occurred. The decrease in S-phase correlates with the observation that high proportion of apoptosis occurs in S-phase cells (Hellström-Lindberg, 1997). The prominent sub-G0 peak represented the apoptotic fraction of the cells on day 14.

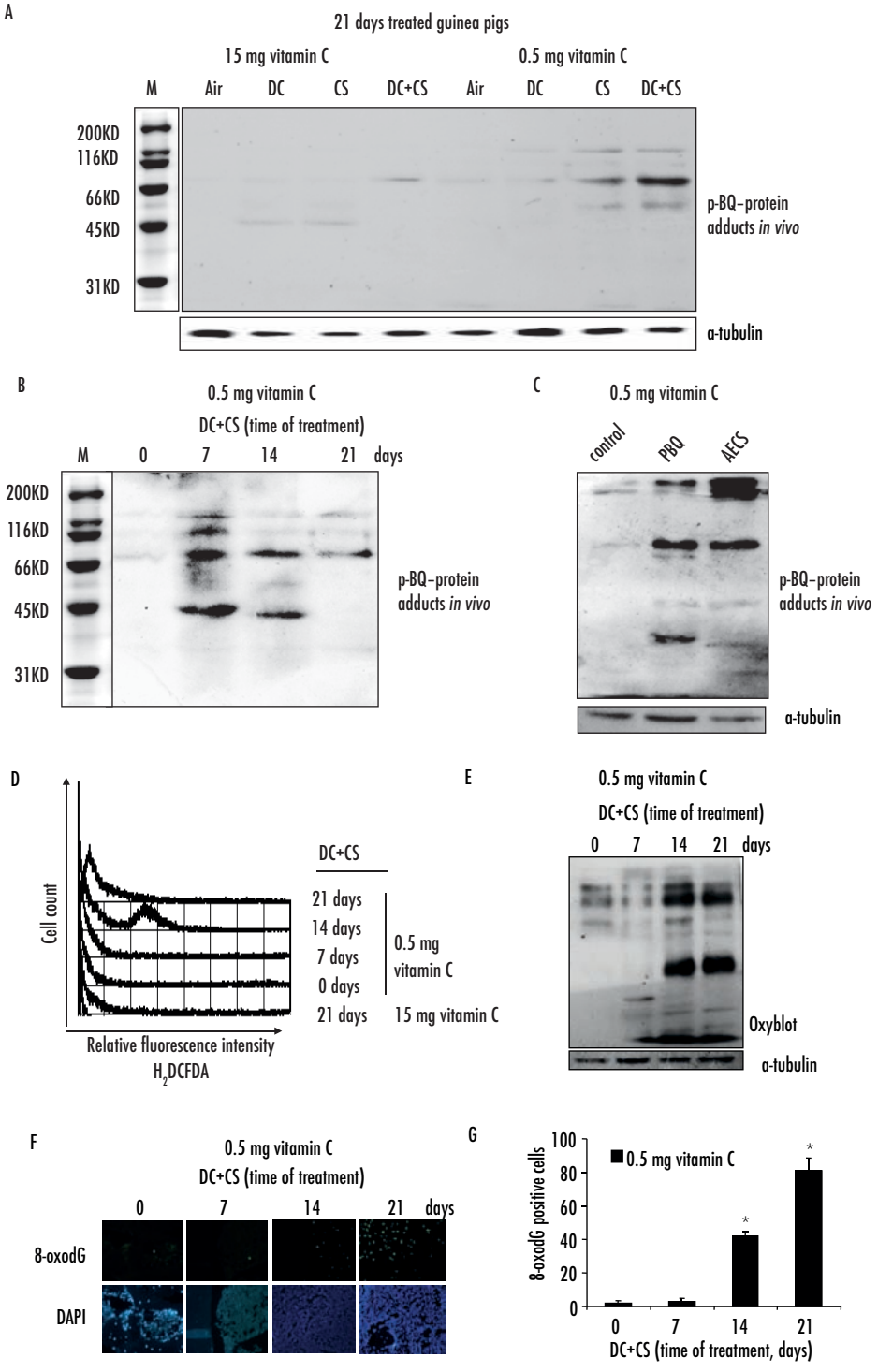
Hematoxylin and eosin staining also showed significant increase ($P<0.05$, n=6) in bone marrow cellularity in MDS guinea pigs. This was corroborated by 4 fold increase of myeloid to non-myeloid ratio ($P<0.05$) (Das *et al.*, 2011). This would indicate that the cells that escaped apoptosis survived and produced hypercellularity and MDS. The net balance of increased proliferation accompanied by apoptosis of the bone marrow may be a mechanism responsible for the paradox of hypercellular bone marrow and ineffective hematopoiesis leading to peripheral blood pancytopenia observed in most cases of MDS.

7.6 Molecular mechanism of cigarette smoke-induced myelodysplastic syndrome and its prevention by vitamin C

7.6.1 p-benzoquinone-induced arylation of proteins

Until now the molecular mechanism of CS-induced MDS is not known. CS contains about 4,000 compounds (Stewart and Kleihues, 2003) which include several carcinogens (Hecht, 1999) and semiquinones (Banerjee *et al.*, 2008; Chatterjee, 2004). The most extensively studied carcinogens are BP and NNK (Stepanov *et al.*, 2006)). However, these compounds are present in smoke at very low concentrations (20-40 ng BP and 80-960 ng NNK/cigarette, respectively) (Hecht, 1999; Stepanov *et al.*, 2006). Moreover, the effects of these carcinogens are not only organ specific but they also produce tumors at very high doses (Hecht, 1999).

Figure 7.7. Identification of p-benzoquinone (p-BQ)-protein adducts and detection of oxidative stress in bone marrow cells of guinea pigs. (A and B) p-BQ protein adducts in the bone marrow cells of cigarette smoke (CS) and dicoumarol (DC)-exposed guinea pigs at different time periods *in vivo*. (C) p-BQ protein adducts formed in *in vitro* after incubation of bone marrow cells with p-BQ and aqueous extract of cigarette smoke (AECS), respectively. (D) Reactive oxygen species (ROS) production in MDS guinea pigs at different time periods, as evidenced by flow cytrometry. The x-axis represents the intensity of ROS-indicator H_2DCFDA. (E) Protein oxidation as evidenced by oxyblot indicating formation of protein carbonyl. (F) DNA oxidation as evidenced by the formation of 8-oxo-7,8-dihydroguanosine (8-oxodG); upper row: green fluorescence indicates formation of 8-oxodG; lower row: stained with 4,6-diamidino-2-phenylindole (DAPI); (magnification 200×). (G) Quantitative evaluation of 8-oxodG; bars over columns represent mean ± standard deviation (n=6), *: significant difference from 0 and 7 days (reproduced from Das *et al.*, 2011).


A
21 days treated guinea pigs
15 mg vitamin C
0.5 mg vitamin C
M
Air
DC
CS
DC+CS
Air
DC
CS
DC+CS
200KD
116KD
66KD
45KD
31KD
p-BQ–protein adducts in vivo
α-tubulin
B
0.5 mg vitamin C
DC+CS (time of treatment)
M
0
7
14
21
days
200KD
116KD
66KD
45KD
31KD
p-BQ–protein adducts in vivo
C
0.5 mg vitamin C
control
PBQ
AECS
p-BQ–protein adducts in vivo
α-tubulin
D
Cell count
Relative fluorescence intensity
H2DCFDA
DC+CS
21 days
14 days
7 days
0 days
0.5 mg vitamin C
21 days
15 mg vitamin C
E
0.5 mg vitamin C
DC+CS (time of treatment)
0
7
14
21
days
Oxyblot
α-tubulin
F
0.5 mg vitamin C
DC+CS (time of treatment)
0
7
14
21
days
8-oxodG
DAPI
G
0.5 mg vitamin C
8-oxodG positive cells
100
80
60
40
20
0
0
7
14
21
DC+CS (time of treatment, days)

We have isolated p-BSQ, a long-lived semiquinone, from CS (Banerjee, 2008; Chatterjee, 2004). *in vivo,* p-BSQ is converted to p-BQ, which is a strong arylating agent (Ghosh *et al.*, 2015; Wang *et al.*, 2006). Using antibody to p-BQ antibody, we had shown formation of p-BQ-protein adducts in bone marrow cells of CS-exposed guinea pigs (Figure 7.7A and B). Formation of p-BQ-protein adducts was also observed after incubation of p-BQ or aqueous extract of CS with bone marrow cells *in vitro* (Figure 7.7C).

7.6.2 p-benzoquinone-induced oxidative damage

p-BQ is also a strong oxidizing agent and causes oxidative damage through formation of ROS (Sullivan and Reynolds, 1976; Valvanidis *et al.*, 2009). ROS leads to the formation of protein carbonyls as well as 8-oxodG that are associated with MDS (Farquhar and Bowen, 2003) and carcinogenesis (Bolton *et al.*, 2000). As shown in Figure 7.7D, ROS increased on the 14th day of CS exposure in bone marrow cells of vitamin C-deficient guinea pigs treated with DC and decreased on day 21 that was prevented by high dose vitamin C (15 mg/d). To further demonstrate the oxidative stress, we have also measured protein carbonyl, an evidence of protein oxidation, and 8-oxodG, an evidence of DNA oxidation (Figure 7.7E and F). As a potent mutagen, 8-oxodG codes for error prone DNA synthesis (Hanes *et al.*, 2006). Elevated levels of 8-oxodG were identified in a considerable proportion of MDS patients (Farquhar and Bowen 2003; Jankowska *et al.*, 2008). Currently, 8-oxodG has become a pivotal biomarker of endogenous DNA oxidation that is linked with initiation and promotion of carcinogenesis (Hwang and Kim, 2007; Valavanidis *et al.*, 2009).

However, how MDS is caused by p-BQ is not clear. The fact that p-BQ derived from CS produces protein adducts and oxidative damage of proteins and DNA would indicate that probably p-BQ is a causative factor of CS-induced MDS. p-BQ is also known to cause damage to tubulin, histone proteins, topoisomerase II, leading to DNA strand break, mitotic recombination and aneuploidy in stem cells or early progenitor cells that may result in MDS (Smith, 1996).

CS-induced MDS is produced only in marginal vitamin C-deficient guinea pigs conjoint with NQO1 deficiency but not in vitamin C-sufficient guinea pigs fed 15 mg vitamin C/d. Mechanistically, vitamin C (extinction coefficient (E´°) = +0.08 V) reduces p-BQ (E´° = +0.71 V) to form less toxic hydroquinone, which is excreted as sulfate and glucuronide. This suggests that apparently the function of vitamin C for prevention of CS-induced MDS is reduction and thereby inactivation of p-BQ.

7.7 Conclusions

CS-induced MDS is a result of the combination of three factors: CS exposure, NQO1 deficiency and marginal vitamin C deficiency. No MDS is produced in vitamin C-sufficient guinea pigs. We therefore consider that human smokers having deficiency of NQO1 conjoint with marginal vitamin C deficiency would be at high risk for developing MDS. However, regular intake of

vitamin C (approximately equivalent to 2 g/adult human/d) should prevent occurrence of MDS in chronic smokers.

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8. Vitamin C, scurvy, inflammation and cancer

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Abstract

In this chapter we do a review about vitamin C, covering aspects of synthesis, absorption, distribution, mechanism of action in the various organs and tissues, and excretion. We also discuss the acute phase response, which is a systemic inflammatory response due to any type of aggression to the body (trauma, infection, cancer, autoimmune diseases), emphasizing the alterations in the serum levels of vitamins occurring during this response, especially vitamin C, that may lead to the onset of diseases due to hypovitaminosis. Finally, we review the use of vitamin C in cancer patients, especially those with hematologic malignancies.

Keywords: vitamins, acute phase response, hypovitaminosis, malignances

Key facts

- Vitamin C is present in significant quantities of fresh fruits, especially citrus fruits and vegetables. It was isolated in the adrenal gland of humans in 1928.
- High levels of vitamin C are found in the pituitary, adrenal glands, white blood cells, ocular tissues, humoral fluids and brain, whereas lower concentrations are present in plasma and saliva.
- Low serum levels of vitamin C are common in patients with severe illness, particularly sepsis associated with multiple organ failure, and is directly related to mortality.
- Recent studies have shown elements of acute phase response (inflammation) in patients with various cancers, including pancreatic cancer, stomach, prostate, esophagus, colon, rectum, non-Hodgkin lymphoma, myeloma and myelodysplasia.
- There are few randomized trials about the use of vitamin C in cancer patients. Patients who participate in these studies should be informed about the risks of using vitamin C, especially intravenously, because there may be toxic effects.

Summary points

- Vitamin C is not synthesized by human beings due to the lack of gulonolactona oxidase enzyme. Food is their sole source of this nutritient.
- The main functions of vitamin C are the synthesis of collagen, the antioxidant action by inactivation of reactive oxygen species, and the action on the vascular tonus by increasing nitric oxide synthesis.
- Individuals with inflammation (acute phase response) have decreased serum levels of vitamin C and other vitamins such as, for example, those of the B complex.
- Scurvy is a disease caused by vitamin C deficiency, and can appear in people with inflammatory response due to increase in vitamin expenditure.
- There is no evidence that vitamin C diminishes the toxic effects of chemotherapy and improves quality of life, or has any antineoplastic action.

Abbreviations

ACTH	Adrenocorticotropic hormone
APP	Acute phase proteins
APR	Acute phase response
CRP	C-reactive protein
CSF	Cerebrospiral fluid
HIF-1	Hypoxia induced factor 1
IFN	Interferon
IL	Interleukin
SVCT	Sodium ascorbate co-transporter
TNF	Tumor necrosis factor

8.1 Vitamin C

Vitamin C, or ascorbic acid, is a water-soluble vitamin playing a vital role as an enzyme cofactor and cellular antioxidant in plants and animals. Primates, including humans, and some species of fish, birds and bats, as well as guinea pigs, do not synthesize vitamin C, because they lack the enzyme gulonolactona oxidase (Chatterjee *et al.*, 1975; Lykkesfeldt *et al.*, 2014; Mandl *et al.*, 2009; Rebouche, 1991). Vitamin C was first isolated by the Hungarian scientist Albert Szent-Györgyi in 1928, and was initially called hexuronic acid. Vitamin C is present in significant quantities of fresh fruits, especially citrus fruits, and vegetables (Carr and Frei, 1999a).

The recommendation of oral intake of ascorbic acid for healthy men and women is 90 and 75 mg, respectively (Institute of Medicine, 2000). After intake and absorption, vitamin C is accumulated in mammalian cells by two types of proteins, SVCTs and hexose transporters. SVCT1 is involved in whole-body homeostasis of vitamin C, while SVCT2 protects metabolically active cells against oxidative stress (Savini *et al.*, 2008; Lykkesfeldt *et al.*, 2014). About 70 to 90% of vitamin C intake is absorbed; however, the absorption decreases approximately 50% when the amount ingested exceeds 1 g per day (Kallner *et al.*, 1979).

When ingested in higher amounts, vitamin C can be metabolized by bacteria in the intestinal lumen, causing eventual diarrhea and abdominal discomfort. The renal excretion is the main mechanism to regulate the amount of body vitamin C in normal conditions, and excretion is proportional to the amount of absorbed vitamin C (Melethil *et al.*, 1986).

The adjustment of the dose-dependent absorption and excretion allows the body to retain vitamin C during low intake and active transport systems allow a wide range of concentrations in different tissues. Vitamin C is present in higher concentrations in the pituitary, adrenal glands, white blood cells, ocular tissues, humoral fluids and brain, whereas lower concentrations are present in the plasma and saliva (Hornig, 1975).

The average life of vitamin C ranges from 8 to 40 days, and is inversely related to the total amount of body vitamin C, which is estimated at around 2 g (Kallner *et al.*, 1979). Severe deficiency is considered when plasma concentrations are below 0.2 mg/dl (Schleicher *et al.*, 2009).

Under physiological conditions vitamin C participates of collagen synthesis by hydroxylation of proline and lysine residues, and synthesis of catecholamines, L-carnitine, cholesterol, and some aminoacids and peptide hormones. In addition, vitamin C is a reducing agent which serves as an antioxidant in oxidative processes mediated by free radicals, and can increase the prooxidant activity of metals such as copper and iron. Normally at low concentrations the effect is a pro-oxidant and at high concentrations, the effect is antioxidant (Mandl *et al.*, 2009). The antioxidant effect occurs in biological fluids by inactivation of reactive species of oxygen and nitrogen, such as hydroxyl and peroxide radicals, superoxide anions and nitrogen dioxide, in addition to hypochlorous acid and peroxynitrite.

Vitamin C increases the absorption of nonheme iron in the gastrointestinal tract by iron reduction to a more easily absorbed form; however, high doses of vitamin C do not increase non-heme iron absorption (Bendich and Cohen, 1990). Therefore, the low intake of vitamin C can be beneficial in conditions where there is body accumulation of iron, as occur in thalassemia and hemochromatosis. Individuals with these diseases have low serum vitamin C, probably due to the interaction of the vitamin with the bulk of existing iron (Carr and Frei, 1999b). A study of two groups of patients on hemodialysis who had refractory anemia and hiperferritinemia showed that intravenous vitamin C administration improved the response to erythropoietin, increased hemoglobin levels in those who received 300 mg of vitamin during each hemodialysis session, over a period of six months. This was probably due to its antioxidant effects and the mobilization of iron their tissue stocks (Attallah *et al.*, 2006).

A review showed that parenteral administration of low doses of vitamin C (200-500 mg/d) and monitoring of serum oxalate levels after the dialysis session is safe. In patients on hemodialysis with refractory anemia and iron overload, parenteral administration of vitamin C improves survival of red blood cells and decreases serum CRP levels (Biesalski, 2008). Carr and Frei (1999b) analyzed study data *in vivo* and *in vitro* with oxidative stress markers specific for DNA, lipids and proteins. *in vitro* studies showed that, with regard to the DNA, vitamin C only acts as an antioxidant when metal ions are not present. In contrast, with regard to lipid peroxidation, vitamin C is an antioxidant, even in the presence of such ions. Data regarding the oxidation proteins suggest that vitamin C does not inhibit the formation of carbonyl radicals (compounds formed from the protein oxidation) in biological fluids. Still, according to these authors, *in vivo* studies indicate that the data on the role of vitamin C in DNA oxidation and protein are inconclusive, and in relation to lipid peroxidation, it acts as an antioxidant. Another important effect of vitamin C is the ability to regenerate vitamin E from its oxidized form, indirectly inhibiting lipid peroxidation (Lykkesfeldt *et al.*, 2014). Recent studies have shown that vitamin C plays an important role in vascular function, increasing nitric oxide synthesis by the action of nitric oxide synthase, leading to vasodilation. This mechanism involves tetrahydrobiopterin, a cofactor of nitric oxide synthase, which is recycled to their oxidized form by vitamin C, and thus sustains

enzyme activity (Lykkesfeldt *et al.*, 2014), besides the modulation of vascular tone having also an antiatherogenic effect on endothelial cells and platelets (Tveden-Nyborg and Lykkesfeldt, 2013). Changes in vascular function are part of a series of events in the systemic response to attacks, such as sepsis bacterial products − that can develop as a consequence of surgery, pneumonia, soft tissue infections associated with cancer or peripheral vascular diseases and products released by cells after tissue injury. The response includes changes characterized by reduction in capillary perfusion, increased vascular permeability, which leads to swelling and arteriolar hypo responsiveness with vasoconstriction and vasodilation (Wilson, 2009). The bacteria killed by antibiotics release large quantities of toxic products, especially the lipopolysaccharide membrane that maintains tissue injury. Septic patients may benefit from treatments to improve vascular function (Wilson, 2009). Low serum levels of vitamin C are common in patients with diseases, particularly sepsis associated with multiple organ failure, and directly related to mortality (Borrelli *et al.*, 1996). Experimental study in rats using endotoxin to trigger acute lung injury was performed to determine if the use of intraperitoneal vitamin C could improve a procoagulant and proinflammatory state caused by lung injury, being checked for mortality, pulmonary capillary leak, the expression of proinflammatory chemokines, and pulmonary microvascular thrombosis. There was improvement in all parameters analyzed after the administration of vitamin C (Fisher *et al.*, 2011). Patients with sepsis of abdominal focus undergoing surgery were treated with vitamin C for six days after the procedure. The quantification of the levels of caspase-3 and of Bcl-2 in neutrophils suggested that vitamin C has had an anti-apoptotic effect in these patients (Ferrón-Celma *et al.*, 2009). A study by Long *et al.* (2003) in trauma patients showed that serum levels of vitamin C were extremely low after the trauma, and that these levels had not improved after supplementation of up to 1000 mg parenterally. The authors suggest the use of a dose of 3,000 mg parenterally in the first three days after severe trauma, because there is an increase in the consumption of the vitamin in these situations. The transport of vitamin C in the central nervous system is complex and has been studied for over half a century (Hammarström, 1966). Vitamin C using injection labeled with carbon-14 in guinea pigs showed that it does not cross the blood brain barrier directly (Harrison *et al.*, 2014).

Studies show that the concentration of vitamin C is higher in CSF than in plasma, suggesting the existence of an active transport in the choroid plexus of this vitamin (Spector and Lorenzo, 1973). There is evidence that glutamate transport is altered in patients with Alzheimer's disease, especially those with sclerosis in the hippocampus. The vitamin C released from astrocytes is responsible for the reuptake of glutamate, which is an oxidizing agent. Therefore, it is assumed that vitamin C protects against cell death. This relationship has been investigated in patients with Huntington's disease also involving cell death (Harrison *et al.*, 2014). The vitamin C ability to prevent dementia and the reduction of age-related cognition need studies with populations with high risk of vascular complications, since lately, vascular comorbidities have been an important factor in age-related dementias. In this context vitamin C presents the following properties: reduced thickness of the intima of the carotid reduced lipid peroxidation and decreased endothelial dysfunction (Harrison *et al.*, 2014).

Vitamin C deficiency causes a disease called scurvy, which was common in sailors and affected about 2 million of them, approximately (Carpenter, 2012). In 1747, English surgeon James Lind conducted what can be considered the first controlled experiment aboard a ship, concluding that oranges and lemons prevent scurvy. He published his findings in 'Treatise on the Scuvy' in 1753 (Léger, 2008).

The signs and symptoms appear about one to three months after the onset of the absence of vitamin in the food, and concentrations between 0.2 and 0.3 mg/dl are usually associated with non-specific symptoms such as fatigue, decreased appetite, and irritability (Smith *et al.*, 2011).

Cutaneous manifestations of scurvy include erosions in the skin, bruises, presence of the corkscrew, hyperkeratosis and perifollicular hemorrhage, these two being pathognomonic findings of the disease. There is also hair loss and poor wound healing (Fossitt and Kowalski, 2014; Reed, 2010; Smith *et al.*, 2011). Joint pain, especially knees, ankles and wrists, and muscle pain are present in about 80% of patients. Bleeding in the joints, especially the hips, knees and ankles are due to bleeding in the synovial vessels and micro fractures (Fain, 2005). There may be ocular manifestations characterized by dry eye syndrome, conjunctival and subconjunctival hemorrhages, and optic nerve sheath (Reed, 2010).

Oral manifestations include hypertrophy and gingival bleeding, halitosis and tooth loss due to the supporting tissue loss. These changes promote a gateway to micro-organisms in the gums. Anorexia and gastrointestinal bleeding may also occur (Reed, 2010; Smith *et al.*, 2011).

Anemia is common and depends on the duration of the disease and in some cases the bleeding is responsible, with an increase in reticulocyte count and with normocitosis and normocromia. Some findings, however, are compatible with hemolysis due to a mean life decreasing of red blood cells, demonstrating extracorpusculares alterations in patients with scurvy. Hemolysis is demonstrated by the increased of unconjugated bilirubin fraction serum levels (Hirschmann and Raugi, 1999).

Cardiopulmonary manifestations include dyspnea, alveolar hemorrhage, fluid retention, anasarca, hemopericardium, myocardial hemorrhage, hypotension, syncope and sudden death (Reed, 2010).

The typical findings of scurvy associated with one of the three following criteria, is called neuropsychiatric scurvy: (1) psychomotor retardation with evidence of affective disorder, such as apathy, anxiety or irritability; (2) extrapyramidal changes; (3) other changes that reveal dysfunctions of the motor nuclei of the base, as catalepsy. The definitive diagnosis is made when there is improvement in signs and symptoms after vitamin C replacement is made (Brown, 2015).

The main differential diagnosis of scurvy should be done with purple diseases caused by trauma, vasculitis, thrombocytopenia, bleeding disorders, amyloidosis, cryoglobulinemia, fat embolism,

etc. The follicular hyperkeratosis due to vitamin A deficiency is another imperative differential diagnosis (Roé *et al.*, 2005).

Hypovitaminosis C, which usually happens due to deficiency of vitamin C intake, can also occur as part of the APR. The vitamin C serum levels in critically ill patients in intensive care units is extremely low, and intravenous injection of IL-2, decreases serum levels significantly. Recent studies suggest that this association is due to tissue redistribution of the vitamin through the circulating leucocytes (Evans-olders *et al.*, 2010). Louw *et al.* (1992) showed that patients with APR have transient yet important decrease in leucocyte vitamin C levels, in addition to decreased serum levels of other vitamins. There are reports of patients with various types of infection, opportunistic or not, the presence of APR, which have decreased serum levels of vitamin C and a clinical diagnosis of scurvy (Burdette *et al.*, 2005; Maltos *et al.*, 2012; Maltos *et al.*, 2011).

Studies have shown elements of APR in patients with various cancers, including pancreatic cancer, stomach, prostate, esophagus, colon, rectum, non-Hodgkin lymphoma, myeloma and myelodysplasia (Alexandrescu *et al.*, 2009; Kotler, 2000). One patient with renal carcinoma with metastases in the liver and the lung treated with high doses of IL-2, developed after six doses, perifollicular hemorrhages, more pronounced in the legs and perimalleolar regions, the corkscrew, and gingival bleeding, weakness, arthralgia and periorbital edema. The platelet count was normal, as well as coagulation tests. Serum vitamin C levels were less than 0.3 mg/dl, and the diagnosis of scurvy was made (Alexandrescu *et al.*, 2009).

8.2 Inflammation

The APR is a systemic inflammatory reaction in the body that occurs in the presence of infections, traumatic tissue injury, surgery, cancer, chronic infections and certain autoimmune diseases. It is believed to be a protective reaction because it removes harmful substances and promotes the repair of tissue injury. The intensity of the response varies with the severity of the lesion and the time of its duration (Bresnahan and Sherry, 2014; Gruys *et al.*, 2005). Once a pathogen is detected or there is a tissue injury due to another cause, as mentioned above, there is recognition of cellular receptors, including Toll-like receptors, which promote a secretion of cytokines, an increase vascular permeability and recruit macrophages and neutrophils for the site, which secrete more cytokines (Bresnahan and Sherry, 2014; Koj, 1996; Kotler, 2000).

Three groups of cytokines can be distinguished: the first is constituted by molecules that act as stimulators or inhibitors of the growth of various cells (IL-2, IL-3, IL-7, IL-10, IL-11, IL-12 and stimulating factor colony growth of neutrophils and macrophages); the second contains the proinflammatory cytokine properties (TNF-α/β, IL-1α/β, IL-6, IFN-α/γ, IL-8 and inhibitory protein macrophage); cytokines with anti-inflammatory activity make up the third group and are represented by IL-1 receptor antagonists, soluble IL-1 receptors, binding protein and TNF-α binding protein IL-1(Gruys *et al.*, 2005). These cytokines – especially IL-1, IL-6, TNF-α and IFN-γ – released together with other inflammatory mediators, diffuse through the extracellular

fluid and flow into the blood stream. Thus, they modulate the immune response leading to complex metabolic disorders (Bresnahan and Sherry, 2014; Gruys *et al.*, 2005). These changes occur throughout the body such as the central nervous system, liver, bone marrow, adrenal gland, etc. Manifestations include fever, which is a result of neuroendocrine changes, with IL-1 and IL-6 (synthesized in the brain stem) being the primarily responsible for its appearance (Gabay and Kushner, 1999; Le Floc'h *et al.,* 2004).

Anorexia is another manifestation, and that may be due to the activity of proinflammatory cytokines in hypothalamic nuclei that control eating behavior, or the activity of other substances, such as leptin secreted by adipocytes, which has a marked effect on feeding behavior and energy balance. It is believed that leptin is the major long-term regulator of body composition (Kotler, 2000). Neuroendocrine changes reflect a complex interaction of various cytokines. There are alterations in the hypothalamic-pituitary-thyroid axis (there is a fall in serum triiodothyronine, suggesting inactivation in peripheral tissues such as the liver), where there is an increase in ACTH secretion with consequent increase cortisol (Gabay and Kushner, 1999). Increased levels of cortisol increase the catabolism of fats, carbohydrates and proteins, being a potential source of energy during severe illness. In addition, it affects the cardiovascular dynamics by fluid retention, and its anti-inflammatory effect can be understood as an attempt to prevent overstimulation of the inflammatory cascade (Boonen and Van den Berghe, 2014; Munck *et al.*, 1984). In severe diseases, the adrenal cortex, despite being activated in response to ACTH, cortisol production, is insufficiently able to activate the glucocorticoid and mineralocorticoid receptors to maintain hemodynamic stability, leading to a relative adrenal insufficiency. Studies show that stimulation with exogenous ACTH generates insufficient increase in cortisol, independent of its basal concentrations, which are typically much higher than in normal individuals (Boonen and Van den Berghe, 2014). The increased secretion of antidiuretic hormone stimulated by IL-6 may be an explanation for the hyponatremia in patients with APR (Gabay and Kushner, 1999).

In APR the body promotes an increase in gluconeogenesis and decreased insulin sensitivity in order to provide organs and tissues that depend predominantly on glucose as a metabolic substrate, such as neurons and blood cells. Young individuals are able to maintain normal glucose levels in stressful situations; however, elderly, obese, those with chronic diseases, receiving drugs that affect insulin sensitivity, or receiving parenteral nutrition, fail to maintain blood glucose levels within normal limits. Hyperglycemia causes cell damage, particularly in those cells which do not require insulin for glucose metabolism, such as hepatocytes, renal tubular cells, endothelial cells, immune cells and neurons (Boonen and Van den Berghe, 2014; Pasquel *et al.*, 2011).

The APR has direct implications for the anemia that occurs in patients. Cytokines and mononuclear phagocytic system cells cause changes in iron homeostasis, proliferation of erythroid precursors in the production of erythropoietin, the mean life of the erythrocytes, and all contribute to the pathogenesis of anemia. Infections, autoimmune diseases and neoplasia activate T cells and monocytes to produce cytokines such as IFN-γ, TNF-α, IL-1, IL-6 and IL-10. IL-6 stimulates the synthesis of hepcidin in the liver, which inhibits the absorption of iron the duodenum and inhibits ferroportin, protein responsible for the release of iron to transferrin in the plasma. The

ferroportin is also inhibited by lipopolysaccharide (LPS) (bacterial endotoxin) and IFN-γ. Another mechanism that contributes to iron sequestration in macrophages is increasing its uptake by the action of IL-10 which increases the levels of soluble transferrin receptors. Furthermore, activated macrophages phagocytose senescent erythrocytes to recycle iron, a process that is mediated by TNF-α (Weiss and Goodnough, 2005). Besides hypoferremia, there are also decreased serum zinc levels, due to the increased metal-binding protein, metallothionein, induced by IL-6 (Gabay and Kushner, 1999).

The APR leads to changes in concentrations of various serum proteins called APP. The functions of these APP vary widely and include binding proteins (opsonins), protease inhibitors, complement factors, apoproteins, fibrinogen, ferritin, acid α1-glycoprotein, CRP, albumin, transthyretin, a protein carrier of retinol, transferrin, haptoglobin and other. Typically there is an increase of some APP and decrease of the other proteins. The group that is has reduced levels are the negative APP, with common transport functions. Part of this group is: albumin, transferrin, transthyretin and the retinol transport protein. The proteins of the other group, which rise opposite to the organic aggressions, are the positive APP, and participate of the defense mechanisms. Therefore, present antiproteasic and antioxidant action and can act as activators of the complement system. Examples of this group are acid α1-glycoprotein, ferritin, fibrinogen, haptoglobin and CRP (Cecilliani *et al.*, 2002; Gruys *et al.*, 2005; Kotler, 2000).

The APR is a normal and desirable occurrence in maintenance of homeostasis of the individual. In this circumstance, common phenomena are needed for full recovery of the individual, however, an excessive response may be maladaptive and cause diseases such as hypoglycemia and acute protein malnutrition, with the presence of hipoalbuminemic edema, severe immune deficiency and tendency to sepsis and poor wound healing including local inflammation, pain, fever, fluid retention aforementioned and metabolic disorders (Kotler, 2000). In addition to typical marasmus (chronic malnutrition) and adult kwashiorkor (acute protein malnutrition), vitamin deficiencies are also common in both biochemical studies (Cunha *et al.*, 2001) such as in reports of cases of scurvy, pellagra, etc. The hypovitaminosis entails biochemical, functional and anatomical changes in various organs and tissues. A deficiency of vitamin A causes xerophthalmia and carotenoid intake can avoid that consequence. Age-related eye diseases such as macular degeneration and cataracts may have its incidence decreased by a rich carotenoids diet, probably due to the reduction of the formation of reactive oxygen species. A study in preschool children with pneumonia, who were previously healthy, showed decreased serum levels of retinol, hemoglobin and albumin. After the recovery of pneumonia, serum retinol levels normalized, suggesting that vitamin A deficiency in these cases may be a manifestation of the APR (Barbosa *et al.*, 2011).

The IL-1 leads to an increased expression of IFN-γ through T lymphocytes, which in turn induces the increase of tryptophan oxidation by activating the indoleamine 2,3-dioxygenase enzyme, increasing niacin synthesis to provide the metabolic demands; this phenomenon leads to a decreased excretion of urinary metabolite of niacin, the N1-methylnicotinamide, with consequent appearance of skin lesions, called flack paint dermatosis, suggestive of deficiency of this vitamin (Le Floc'h *et al.,* 2004; Maltos *et al.,* 2015).

Excess body weight and obesity (BMI from 25 to 29.9 kg/m^2 and greater than 30 kg/m^2 body surface, respectively) are considered changes in nutritional status. According to the WHO obesity has more than doubled since 1980, and in 2014 more than 1.9 billion adults (over 18 years age) were overweight, and of these, 600 million were obese. In 2013, 42 million children less than five years were overweight or were obese (WHO, 2015). Body weight excess is a major contributor to chronic diseases, including type 2 diabetes, cardiovascular diseases, and some cancers (Kopelman, 2000). Different types of immune cells are recruited by adipose tissue under physiological conditions, indicating that these cells contribute to their regulation. One of the main stimuli is the release are free fatty acids through lipolysis, in both physiological (e.g. cold), and pathological conditions (e.g. type 2 diabetes). The increase of adipose tissue promotes the infiltration of immune cells in tissue remodeling, a process which promotes inflammation of that tissue. The release of pro-inflammatory cytokines stimulates lipolysis and causes insulin resistance, leading to dysfunction of adipose tissue metabolism and systemic changes (Grant and Stephens, 2015). Only TNF-α has a pronounced lipolytic effect in human adipocytes, although other cytokines are released (Arner and Langin, 2014).

Cancer patients have an increased lipolysis *in vivo*, and studies show that these patients lose fat before muscle wasting; therefore, the weight loss is not explained only by increased energy expenditure (Bing and Trayhurn, 2008; Tisdale, 2005). A recent review conducted in the UK showed a positive association between obesity and the incidence of various cancers, including leukemia (Bhaskaran *et al.*, 2014). Three meta-analyses suggest that overweight and obesity can be associated with an increased risk of incidence of non-Hodgkin's lymphomas, various types of leukemia and multiple myeloma (Larsson and Wolk, 2007; Larsson and Wolk, 2008; Wallin and Larsson, 2011). A study conducted from January 1999 to December 2008 with 469 patients treated for acute myeloid leukemia, showed that 81% of those with acute promyelocytic leukemia (M3 in the French-American-British classification, which classifies acute myeloid leukemia M1 to M7) were obese. The authors conclude that the M3 leukemia and metabolic syndrome may have a common pathogenic pathway involving the retinoic acid receptors (Tedesco *et al.*, 2011). In addition, obesity has shown adverse effect on the results of treatment in children with acute myeloid leukemias, especially M3 (Lee *et al.*, 2012). In according to Castillo *et al.* (2012) besides the higher incidence of acute leukemia in obese adults, mortality in these patients is also higher. Obese patients are at increased risk for certain types of cancer, however, the molecular mechanisms responsible for this association still need to be clarified. They include hormonal and growth factors related to obesity, modulation of energy balance and calorie restriction, multiple cell signaling pathways and inflammatory processes that promote change and the proliferation of cancer cells (Park *et al.*, 2014; Vucenik and Stains, 2012).

8.3 Vitamin C and cancer

In 1976 Ewan Cameron and Linus Pauling claimed that vitamin C free availability was an important factor in organic resistance, based on evidence that cancer patients had an increase in consumption of this vitamin when compared with normal subjects, and published evidence

that vitamin C could lead to remission in advanced cancers in humans (Cameron and Campbell, 1974; Cameron *et al.*, 1975). Cameron and Pauling (1976) administered high doses of intravenous vitamin C (10 g/d) in patients with various types of cancer, in terminally state, and found an increased survival rate, about three times higher when compared with a control group under the same conditions, i.e. patients with many types of malignancies, also in terminally state. Subsequent studies have shown the same results, however, vitamin C was used orally (Creagan *et al.*, 1979; Moertel *et al.*, 1985).

Numerous studies have shown a decrease in vitamin C serum levels in patients with cancer and their correlation with mortality (Fain *et al.*, 1998; Mayland *et al.*, 2005). Anthony and Schorah (1982) quantified 158 vitamin C samples in plasma and leucocytes of 139 patients with lung cancer at various stages of disease, and found that most samples showed (64% plasma and 25% in mononuclear leucocytes) values about three times smaller than the reference values. Yet, according to these authors, since there is evidence that immune mechanisms influence on the tumor growth and the emergence of metastases in man, the effect of vitamin C supplementation should be tested in patients with lung cancer. In addition to antioxidant activity, other mechanisms such as inhibition of enzymes to eliminate carcinogens and suppression of abnormal proliferation associated with precancerous lesions, are associated with vitamin C (Wargovich, 1997).

Epidemiological studies suggest that foods rich in vitamin C have a protective role against the development of cancer and the serum levels of this vitamin are inversely proportional to the risk of cancer; however, large randomized trials comparing supplements containing antioxidant vitamins (A, C, E and beta carotene) showed no protective effects (Du *et al.*, 2012). It is important to consider the actual amount of vitamin C in the treatment of cancer due to factors such as route of administration (intravenous route provides plasma concentrations about 70 times greater than the oral route); high concentrations of vitamin C administered intravenously generate hydrogen peroxide in the extracellular fluid, which are anticancer. Recent studies show anticancer effects following intravenous administration of high doses of vitamin C (Parrow *et al.*, 2013).

The HIF-1 is a molecule that responds to hypoxia, metabolic stress and allows the cell to adapt to environmental stress by increasing glycolysis, angiogenesis, erythropoiesis, and tissue remodeling. It is a critical factor for normal tissue function, and its activation is implicated in a number of pathologies. The solid tumor microenvironment leads to its activation, causing tumor growth, metastasis, chemo and radio resistance. *in vitro* studies have shown that vitamin C supplementation inhibits the HIF-1 in neoplastic cells (Kuiper *et al.*, 2014).

In vitro, high concentrations of vitamin C induce apoptosis in several leukemic cell lines, for the generation of hydrogen peroxide, but not on normal hematopoietic progenitor cell lines. Furthermore, these high concentrations inhibit neoangiogenesis in immunodeficient mice (Parrow *et al.*, 2013). These authors noted that in leukemic cells, the HIF-1 transcription is suppressed and the exposure to high doses of vitamin C markedly increases the content of this vitamin in these cells and inhibits nuclear translocation of nuclear factor κB, which mediates the expression of HIF-1. There is a close relationship between leukemia and oxidative stress.

Leukemic cells produce large amounts of reactive oxygen species compared to normal cells. Patients with chronic leukemia show significant increase in leucocyte hydrogen peroxide and malonic dialdehyde serum levels when compared to normal controls (Al-Gayyar *et al.*, 2007). Vitamin C, when combined with irradiation, does not inhibit the cytotoxic effect of it in human promyelocytic leukemia cells (HL-60), although this effect mildly decreases in intracellular reactive oxygen species (Terashima *et al.*, 2013).

Amongst acute myeloid leukemia, the M3 type responds well to therapy with arsenic trioxide, unlike the other six types. A study combining chemotherapy with arsenic trioxide followed by intravenous administration of vitamin C in acute myeloid leukemias, except for the M3, showed improved chemotherapeutic action of arsenic trioxide (Aldoss *et al.*, 2014). The virus of linfotropic human T cell (HTLV-1) is a retrovirus that causes T cell leukemia. Harakeh *et al.* (2007) have shown that vitamin C has an antiproliferative effect on T-cell leukemia, and this effect is dose and time dependent. Moreover, a study conducted in patients with myeloma showed that administration of vitamin C inhibits cytotoxicity induced by bortezomib against neoplastic cells *in vitro* (Perrone *et al.*, 2009). In hematopoietic stem cell transplantation there is a decrease of vitamin C serum levels throughout the acute phase, and this deficiency is associated with the CRP and ferritin increase. Under these conditions the supplementation of vitamin C seems promising (Berger and Oudemans-van Straaten, 2015).

A recent systematic review and meta-analysis showed no evidence that there is an antineoplastic action of vitamin C, whether oral, intravenous, or a combination of the two routes of administration in cancer patients. The evidence that intravenous vitamin C decreases the toxicity of chemotherapy and improves quality of life, is small. The review also identified potential toxic effects of using this vitamin intravenously. There are few randomized studies that approach this issue. Patients should be informed about these risks and participate only in high quality trials (Jacobs *et al.*, 2015).

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Iron in health of blood diseases

9. Iron supplementation among low-income people: evidence from randomized controlled trials

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Abstract

Iron deficiency anaemia is a preventable cause of morbidity affecting millions of children and adults in poor communities of both developing and developed countries. Measurement of iron status is not always an economically viable option especially in developing countries. Long-term iron supplementation daily for 15 months in poor infants and young children was found safe in community-based controlled trials in Bangladesh. Another large community-based controlled study targeting low-income postpartum women in Mississippi showed effectiveness of a universal iron supplementation strategy in improving haemoglobin status and reducing the prevalence of anaemia.

Keywords: children, postpartum women, morbidity, growth, haemoglobin

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Key facts

- Long-term oral supplementation of iron in infants and young children is safe.
- Poor children in Bangladesh suffer from several episodes of respiratory infections and diarrhea every year.
- Iron supplementation did not improve growth of malnourished children in Bangladesh, possibly because they often suffer from infections.
- The majority (52%) of the low-income women after delivery were anaemic.
- More than one-third of the women after delivery had a double burden of anaemia and obesity in Mississippi.

Summary points

- The children in Bangladesh suffered more often from acute respiratory infections than diarrhoea or dysentery (five vs. three episodes per child per year).
- The children treated with iron and vitamins (treatment group) and the controls who were treated with vitamins only did not differ in the number of episodes, average duration of each episode or total days of illnesses.
- A 49% greater number of episodes of dysentery was observed with iron supplementation in a subset of young infants.
- Giving daily oral iron supplementation did not improve growth of malnourished children in Bangladesh.
- A strategy of universal iron supplementation to all low-income women after delivery, irrespective of their iron status, improved their haemoglobin levels and decreased the prevalence of anaemia at six months of follow-up.

Abbreviations

ARI	Acute respiratory tract infection
CDC	Centers for Disease Control and Prevention
Hb	Haemoglobin
WIC	Special supplemental nutrition program for women, infants, and children

9.1 Introduction

Iron deficiency anaemia is a preventable cause of morbidity affecting 30 to 50% of the world population (WHO, 2007a). The WHO estimates that some two billion people are anaemic, and that majority are due to iron deficiency (WHO, 2007b). In the USA, about one in ten women of childbearing age is iron-deficient, and one in twenty is iron-deficient and anaemic (Looker *et al.*, 2002). Consequences of iron deficiency on human health are many, including increased risk of death of anaemic women and their infants during the perinatal period, delayed or impaired mental and physical development of children, reduced cognitive functions of young children, and reduced physical work capacity and pro-ductivity of manual workers (Hamid Jan *et al.*, 2010; WHO, 2007b). However, there are conflicting reports concerning the beneficial effects of iron supplementation on growth and development of children (Iannotti *et al.*, 2006). Some studies have shown improved development of children after iron supplementation (Black *et al.*, 2004; Idjradinata and Pollitt, 1993; Lind *et al.*, 2004; Soewondo *et al.*, 1989; Stoltzfus *et al.*, 2001), whereas a couple of studies found no beneficial effects of short-term (Lozoff *et al.*, 1982) or long-term (Lozoff *et al.*, 1996) iron supplementation on infant development. Our group also did not find any beneficial effect of long-term oral iron supplementation on growth (Rahman *et al.*, 1999) and morbidity (Mitra *et al.*, 1997) of children in a poor community of Bangladesh. However, the long-term low dose iron supplementation in children was safe (Mitra *et al.*, 1997).

Iron requirements are increased in women during pregnancy due to the expansion of blood volume and growth of the foetus and placenta (Institute of Medicine, 2001). Routine iron and folate supplementation during pregnancy improves maternal health and pregnancy outcomes. Women who receive daily antenatal iron supplementation are more likely to increase their Hb and serum ferritin levels during pregnancy and also improve the iron status in the puerperium (Müngen, 2003). A systematic review and meta-analysis of 48 randomized trials and 44 cohort studies suggests that prenatal iron supplementation is significantly associated with a decrease in the risk of low-birth-weight babies (Haider *et al.*, 2013).

Although maternal haematological status is expected to return to normal after delivery, some women are still at risk of developing postpartum anaemia (CDC, 1998). These risk factors include anaemia continued through the third trimester of pregnancy, excessive blood loss during delivery, and multiple births (CDC, 1998). The risk of postpartum anaemia is much higher among low-income women. Among women with household income being less than 130% of poverty, the prevalence of anaemia was 30, 20, and 16% at 0-6, 7-12, and 13-24 months after delivery,

respectively (Bodnar *et al.*, 2002). Again, the women who participated in the WIC Program, the prevalence of postpartum anaemia was approximately 27% (Bodnar *et al.*, 2001).

The risk of anaemia and iron deficiency is reported higher among the poor. This book chapter focuses on results of three community-based randomized iron supplementation trials conducted by our team in Bangladesh and in Mississippi: (1) to evaluate the effect of long-term iron supplementation on morbidity of infants and young children in a poor community of Bangladesh (Mitra *et al.*, 1997); (2) to examine the effect of long-term iron supplementation on growth of malnourished children in Bangladesh (Rahman *et al.*, 1999); and (3) to evaluate the effect of iron supplementation among low-income postpartum women who participated in the WIC Program in Mississippi (Mitra and Khoury, 2011).

9.2 Iron and morbidity in children

A double-blind controlled trial was conducted from January 1990 through December 1991 among 349 children, aged 2-48 months, living in a peri-urban slum community of Nandipara, about 10 km northeast of Dhaka, Bangladesh (Mitra *et al.*, 1997). This area was a low-lying village often flooded by river water. The inhabitants were very poor with an average household income of $50 per month. The villagers seek health care from a local day-care clinic run by the International Center for Diarrhoeal Disease Research, Bangladesh and from village practitioners. The total population of the village was 3,712, with 550 children below five years of age.

9.2.1 Case definitions

Diarrhoea was defined as three or more liquid or watery stools in a 24-hr period. The presence of blood and/or mucus in any stool during the episode was considered dysentery. ARI was defined as cough (or difficulty in breathing) and fast breathing, with or without chest indrawing. Three or more symptom-free days differentiated episodes of illnesses.

9.2.2 Supplementation and morbidity surveillance

Each eligible child was randomly assigned to one of the two treatments: the test (iron plus vitamins), and the control (vitamins only). The dose of iron was 125 mg of ferrous gluconate containing 15 mg of elemental iron. Mixture of vitamins included vitamin A (80,000 RE/l, vitamin D (2 mg cholecalciferol/l), and vitamin C (10 g/l). Mothers were advised to administer the medicine in syrup form daily for 15 months. Trained community health workers (one for each household cluster) visited their assigned houses daily to monitor compliance and to collect data on morbidity of diarrhoea, dysentery and respiratory infections, any referrals, or any deaths. Two study physicians attended the weekly clinic and provided treatment for any ailments. The investigators also made unscheduled spot visits to monitor the activities of the workers and to detect any unusual events.

9.2.3 Results and discussion

Diarrhoea

Bangladesh is a diarrhoea-prone country. On an average, each child in this study suffered from about three episodes of diarrhoea per year, and each episode lasted for three days. The total days of illness due to watery diarrhoea was almost double in young infants compared to that in older children (13-17 vs. 5-7 days, respectively) (Table 9.1). However, there was no significant difference in watery diarrhoea between the test and the control groups.

Dysentery

The average number of episodes of dysentery was similar to that of watery diarrhoea. On an average, dysenteric illnesses lasted for four days, and the total days of illnesses per child per year was also longer in case of dysentery than that of watery diarrhoea. It was interesting that young infants in the test group suffered from significantly more episodes of dysentery (P=0.03) and more days of illness from dysentery per child per year (P=0.02) compared to the controls (Table 9.2).

Acute respiratory tract infection

The children suffered more often from respiratory infections than diarrhoea (five vs. three episodes per child per year). Each episode of respiratory infection lasted for five days and each child suffered from a total of 24 days from ARI. The two treatment groups did not differ in terms of number of episodes, duration of each episode, and the total days of illness per child per year from ARI (Table 9.3).

Table 9.1. Effect of iron supplementation on watery diarrhoea in children.[1]

	<12 months			12-48 months		
Characteristic	**Iron (n=30)**	**Control (n=41)**	***P*-value [2]**	**Iron (n=88)**	**Control (n=90)**	***P*-value [2]**
No. episodes per child per year	4.2 (2.6, 7.1)	3.7 (2.2, 6.4)	0.44	2.5 (1.6, 4.0)	2.2 (1.5, 4.0)	0.27
Days of illness per child per year	16.7 (8.5, 27.0)	12.7 (6.3, 21.2)	0.26	6.9 (2.6, 13.3)	5.2 (3.1, 10.2)	0.27
Duration of episode (days)	3.2 (2.3, 4.1)	3.3 (2.3, 4.5)	0.89	2.5 (2.0, 3.2)	2.3 (2.0, 3.0)	0.27

[1] Median and quartiles are shown.

[2] Mann-Whitney U test between iron and control groups.

Table 9.2. Effect of iron supplementation on dysenteric illnesses in children.[1]

	<12 months			12-48 months		
Characteristic	**Iron (n=34)**	**Control (n=44)**	***P*-value [2]**	**Iron (n=102)**	**Control (n=103)**	***P*-value [2]**
No. episodes per child per year	5.2 (2.4, 7.8)	3.5 (2.1, 4.8)	0.03	2.0 (0.8, 4.0)	2.4 (0.8, 5.1)	0.54
Days of illness per child per year	22.2 (10.4, 41.0)	14.3 (6.3, 22.2)	0.02	8.1 (3.2, 16.8)	9.6 (3.0, 22.9)	0.34
Duration of episode (days)	4.4 (3.2, 6.4)	4.0 (2.8, 5.7)	0.39	4.0 (3.0, 5.5)	4.3 (3.0, 6.1)	0.56

[1] Median and quartiles are shown.

[2] Mann-Whitney U test between iron and control groups.

Table 9.3. Effect of iron supplementation on acute respiratory tract infections in children.[1]

	<12 months			12-48 months		
Characteristic	**Iron (n=32)**	**Control (n=44)**	***P*-value[2]**	**Iron (n=109)**	**Control (n=104)**	***P*-value[2]**
No. episodes per child per year	7.5 (4.9, 9.7)	6.9 (4.8, 9.7)	0.74	4.7 (3.0, 6.9)	4.4 (2.2, 7.0)	0.53
Days of illness per child per year	34.7 (19.7, 76.8)	39.4 (22.0, 65.0)	0.97	19.3 (10.2, 37.9)	20.4 (7.3, 34.7)	0.93
Duration of episode (days)	5.5 (3.6, 8.1)	5.1 (3.8, 7.7)	0.97	4.5 (2.8, 6.1)	4.7 (3.0, 6.1)	0.56

[1] Median and quartiles are shown.

[2] Mann-Whitney U test between iron and control groups.

In this study, long-term iron supplementation was safe in most children. Episodes and duration of diarrhoea and ARI were not increased after iron supplementation. However, a subset of young infants showed a significant increase in number of episodes and duration of dysentery compared with the control group. The possible mechanism of this increase of dysenteric illnesses could be due to the available free iron which helps in multiplications of bacteria including *Candida*, *Escherichia*, *Mycobacterium*, *Pasteurella*, *Shigella*, and *Staphylococcus* (Weinberg, 1974). It is likely that iron enhanced growth of *Shigella* bacteria causing dysentery in young infants who were not able to metabolize iron as efficiently as did older children.

9.3 Iron and growth in children

Children, aged 6-71 months, were part of this study (Rahman *et al.*, 1999) as the study described earlier (Mitra *et al.*, 1997). Therefore, they were randomly assigned to either a test group receiving iron (125 mg of ferrous gluconate) plus vitamins or the comparison group receiving vitamins only. These children were measured for their weight and length (or height for children >24 months old) once a month for 12 months.

Of the 250 children enrolled, 27 (10.8%) were lost to follow up. The reason for dropout was out-migration and one death due to drowning. The weight and height gains did not differ between the two groups after adjusting for age (Table 9.4). There was also no difference in weight and height gains in the two groups when boys and girls were analysed separately.

9.4 Iron supplementation among low-income postpartum women in Mississippi

9.4.1 Study population

This study by Mitra and Khoury (2011) was conducted in Mississippi from June 2003 to September 2007 among 959 girls and women aged 13 years and older, between two and six weeks after delivery, and certified for WIC. Those with Hb <7.0 g/dl or with sickle cell anaemia were excluded.

Table 9.4. Weight and height increments of Bangladeshi children aged 6-71 months receiving iron supplementation plus vitamins (intervention) or vitamins only (comparison).[1]

Variable	Intervention	(n=107)	Comparison	(n=116)
Weight increment, kg	1.63±0.77	(n=9)	1.47±0.66	(n=18)
<12 month	1.73±0.50	(n=25)	1.69±0.47	(n=24)
12-23 month	1.34±0.92	(n=22)	1.36±0.56	(n=26)
24-35 month	1.13±0.43	(n=51)	1.23±0.47	(n=48)
All	1.35±0.65	(n=107)	1.39±0.54	(n=116)
Height increment, cm	7.9±2.1	(n=9)	8.07±2.1	(n=18)
<12 month	6.70±1.21	(n=25)	6.48±1.26	(n=24)
12-23 month	5.94±1.37	(n=22)	6.14±1.36	(n=26)
24-35 month	5.30±1.00	(n=51)	5.35±1.00	(n=48)
All	6.01±1.47	(n=107)	6.18±1.58	(n=116)

[1] Values are mean ± standard deviation.

9.4.2 Randomization

There were three treatment arms of this study: (1) control – women identified as 'high risk' based on the current CDC guidelines were screened for Hb status; (2) group I – all participants in this group were screened for Hb status; (3) group II – all participants in this group were given iron treatment, irrespective of their Hb status. 11 health clinics which were participating community partners in this study were randomly assigned to one of these three treatment arms. All participants of the same clinic received the same type of treatment (either control, n=247; group I, n=364; and group II, n=348). Hb levels and body weight and height were measured at enrolment and at six months of follow up.

9.4.3 Interventions

Control (selective screening): women at 'high risk' were defined by anaemia continued through the third trimester of pregnancy, excessive blood loss during delivery, and multiple births (CDC, 1998). These 'high risk' women were screened for anaemia (Hb <12.0 g/dl). Anaemic women were given iron tablets containing 325 mg ferrous sulphate (65 mg of elemental iron), and instructed to take one to three tablets daily for two months according to their Hb concentration: one table daily for Hb >10.0 but <12.0 g/dl; two tablets daily for Hb 9.0 to 10.0 g/dl; and three tablets daily for Hb 7.0 to <9.0 g/dl.

Group I (universal screening): all women in this group were screened for anaemia, and iron was given as in the control group for two months.

Group II (universal supplementation): all women in this group were given iron, one tablet daily for two months, irrespective of their Hb values.

9.4.4 Results and discussion

Based on the Hb status at enrolment, a large proportion (52%; 501/959) of postpartum women were anaemic. 33% (203/617) were still anaemic at six months postpartum who needed further supplementation. More than one-third of the study participants had a double burden of anaemia and obesity at enrolment. This reflects the overall picture of Mississippi, which is one of the US states with the highest incidence of obesity.

Of the three treatment groups, group II, which received universal iron supplementation irrespective of the Hb status had significantly improved on the Hb status ($P<0.001$) and had the lowest proportion of anaemic women ($P<0.001$) at six months postpartum after intervention (Table 9.5). The proportion of reduction of anaemia from baseline to six months postpartum period was 20, 10 and 27 percentage points among women in the control group, group I, and group II, respectively ($P<0.001$).

Table 9.5. Haemoglobin status and women with anaemia in the three treatment groups.

Characteristics[1]	Control	Group I	Group II	*P*-value[2]
Hb at enrolment (g/dl), mean ± SD	11.7±1.8, n=247	11.8±1.6, n=364	11.8±1.6, n=348	0.58
Hb at six months postpartum (g/dl), mean ± SD	12.2±1.3, n=162	12.0±1.4, n=219	12.7±1.4, n=236	<0.001[3]
Women with anaemia at enrolment, n (%)	134/247 (54.3)	194/364 (53.3)	173/348 (49.7)	0.48
Women with anaemia at six months postpartum, n (%)	55/162 (34.0)	95/219 (43.4)	53/236 (22.5)	<0.001
Women with anaemia and obesity at enrolment, n (%)	96/247 (38.9)	143/364 (39.3)	125/348 (36.9)	0.71

[1] Hb: haemoglobin; SD: standard deviation; obesity was defined as body mass index >30.0 kg/m^2.

[2] Tukey's HSD test.

[3] Control vs. group I: P=0.25; control vs. group II: P=0.002; group I vs. group II: P<0.001.

The side effects of iron supplementation were minimum, and did not appear to be present a barrier to the participants. At follow-up, only 48/617 (8%) women mentioned gastrointestinal symptoms and only 32/617 (5%) reported that they had forgotten to take the tablets daily. This contradicts the common belief that women stop taking iron tablets mainly due to side-effects. This study clearly demonstrated that a universal iron supplementation strategy was effective in reducing the prevalence of anaemia among low-income postpartum women.

9.5 Conclusions

Based on our studies in Bangladeshi children and postpartum women in Mississippi, iron given daily for long term is safe. A large proportion of poor community people in both developing and developed countries present with iron deficiency and they can be treated with oral iron safely for a longer term. Policy makers may revisit the current policy of selecting high-risk postpartum women for anaemia screening and iron treatment.

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10. Strategy to reduce iron-deficiency anemia in childhood: fortification of drinking water with iron

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Abstract

Iron deficiency anemia is considered an important public health issue due to its elevated prevalence and the consequences of it in short and long term. The most vulnerable groups are children and pregnant women. It is explained by their higher need for iron and also by the lower intake of this mineral in their diet. One of the strategies to increase iron intake and to prevent and/or reduce anemia is food fortification. This strategy is considered as the most practical and cost-efficient solution mainly in regions where high prevalence of iron deficiency anemia is found. In Brazil the fortification of corn and wheat flour is mandatory. The goal is to increase availability of iron-rich food to the Brazilian population, thus it contributes to reduce the prevalence of iron deficiency anemia. However, the prevalence of anemia continues to be high in the country. The addition of iron to drinkable water can prevent deficiency. Besides drinking, drinkable water is used in food preparation, which helps to elevate iron intake even more. Many studies developed in various regions in Brazil used water as the vehicle of fortification. These studies worked on different concentrations of iron and vitamin C. The results affirm that drinkable water iron fortification is an important strategy to reduce iron deficiency anemia and should be considered as a viable measure of prevention to specific groups that present higher vulnerability.

Keywords: iron status, iron carrier, control, prevention

Key facts

- A lack of iron in the diet may result in the development of iron deficiency anemia.
- Iron is an essential mineral critical for motor and cognitive development. Children and pregnant women are especially vulnerable to the consequences of iron deficiency.
- Globally, anemia reaches 1.62 billion people, which corresponds to 24.8% of the population. Brazil, estimated numbers show that anemia affects 54.9% of the children preschool.
- Iron fortification of foods is considered the most effective method to fight anemia.
- The addition of iron to potable drinking water is one alternative to the control and prevention of iron deficiency and anemia.

Summary points

- Iron deficiency is the result of a negative balance between the amount of biologically available iron consumed and the body's iron requirements over a long period of time.
- The typical dietary patterns and the high physiological iron requirements of children under five years of age are the main factors responsible for the greater vulnerability to anemia.
- Food fortification requires addition of iron to processed food. It is a good strategy that can be lead to relative rapid improvement of the iron status of the population.
- Drinking water, other than used for drinking, is commonly used for preparation of foods, which may contribute even more towards increasing iron ingestion by its fortification.
- The association between iron sulfate and vitamin C promotes more stability to the iron salt stopping its oxidation. It minimizes flavor, odor and turbidity changes in water after fortification.

Abbreviations

DALYs	Disability-adjusted life years
EDTA	Ethylenediaminetetraacetic acid
Hb	Hemoglobin
RNI	Recommended nutritional intake

10.1 Introduction

Anemia is a condition where the number of red blood cells is insufficient to meet the physiological needs of the body. Physiological needs vary according to age, gender, altitude and physiological conditions such as pregnancy. Iron deficiency is described in the literature as the most common cause of anemia, however anemia can be associated with other nutritional deficiencies, including folate, vitamin B12 and vitamin A. Moreover, it can be associated with pathological conditions such as acute and chronic inflammation, parasitic infestation and hereditary or acquired diseases that affect the synthesis of Hb (WHO, 2011).

According to the WHO, iron deficiency anemia was identified as one of the ten main risk factors associated with morbidity and mortality in underdeveloped and developing countries (WHO, 2008). Globally, anemia reaches 1.62 billion people (95% confidence interval: 1.50-1.74 billion), which corresponds to 24.8% of the population. The highest prevalence was found in preschool children (47.4%; 95% confidence interval: 45.7-49.1%). In Brazil, estimated numbers show that anemia affects 54.9% of the children at that age (WHO and CDC, 2008).

A study at a global, national and regional level about the concentration of Hb and the prevalence of anemia in children, pregnant women and non-pregnant women pointed a reduction of anemia during the period of 1995 to 2011. Nevertheless, despite the modest improvement in that period, the concentration of Hb remains low and the prevalence of anemia high in the poorest regions in the world. It represents an obstacle to the reduction of maternal and neonatal mortality and also to a healthy development during infancy at its early stages (Stevens *et al.*, 2013).

Iron deficiency in children is a result of the unbalance between iron storages at birth, iron intake and/or iron absorption, and the needs due to growth and blood loss (Oski, 1989). At this age range, the factors involved in the etiology of iron anemia are: low birth weight and prematurity, due to lower iron storages at birth and higher iron needs; inadequate nutrition such as the premature ingestion of cow's milk and/or solid food, which have lower bioavailability when compared to breast milk; and late introduction of iron-rich foods in addition of insufficient intake and mineral loss (Olivares and Walter, 2004).

The eating patterns combined with high physiological iron needs make children vulnarable for development of anemia . About 80% of iron deficiency cases have as a causal factor the insufficient intake and the low bioavailability of iron in food (Tudisco, 1988). Studies have shown that

bioavailable iron present in diets is not enough to maintain the adequate nutritional status of iron, which leads – consequently – to anemia in children (Fomon *et al.*, 2005; Zimmermann *et al.*, 2005).

The consequences of iron-deficiency anemia in children have been studied for more than 25 years. Changes in the development and behavior have been consistently documented. In 1978 Oski and Honig (1978) did one of the pioneer studies related to behavior alterations. Significant behavior differences and an also significant improvement on the mental development index were found between treated children and anemic children. Many other studies found a relation between the cognitive and motor development delay in anemic children (Grantham-Mcgregor and Ani, 2001; Kariger *et al.*, 2005). Another important point is that these children, even after five (Lozoff *et al.*, 1991) or ten years (Lozoff *et al.*, 2000) of treatment for anemia, showed some damage in their development.

Besides the cognitive, behavioral and motor changes related to anemia, other consequences observed are: reduction of physical work performance; alterations at cellular immunity and at the bactericidal capacity of the neutrophils; higher susceptibility to infections, mainly in the respiratory tract; functional and histological modifications in the digestive tract; and lack of hepatic vitamin A recruitment (Olivares and Walter, 2004).

It is important to take into consideration that the intensity of anemia effects in children's development may vary depending on severity, duration and age when anemia takes place (Walter, 1996). According to WHO mortality data, around 0.8 million deaths (1.5% of the total) can be attributed to iron deficiency each year, and a similar number to vitamin A deficiency. In terms of the loss of healthy life, expressed in DALYs, iron-deficiency anemia results in 25 million DALYs lost (or 2.4% of the global total) (WHO and FAO, 2006a).

In regions where the prevalence of iron-deficiency anemia is high, control measures at individual level tend to be ineffective. Thus, fortification of foods with iron can be considered the most effective method due to its low cost, not depending on individual decision and being able to be addressed to all population sectors (WHO and FAO, 2006b).

10.2 Fortification of foods

Food fortification is usually regarded as the deliberate addition of one or more micronutrients to particular foods, so as to increase the intake of these micronutrient(s) in order to correct or prevent a demonstrated deficiency and provide a health benefit. A concern with nutritional deficiencies in populations and utilization of fortification as an intervention measure were extensively documented throughout the twentieth century. Food fortification has a long history of use in industrialized countries for the successful control of deficiencies of vitamins A and D, several B vitamins (thiamine, riboflavin and niacin), iodine and iron. Salt iodization was introduced in the early 1920s and has since expanded progressively all over the world to the extent that iodized salt is now used in most countries. From the early 1940s onwards, the fortification

of cereal products with thiamine, riboflavin and niacin became common practice. Margarine was fortified with vitamin A and milk with vitamin D. Foods for young children were fortified with iron, a practice which has substantially reduced the risk of iron-deficiency anemia in this age group. In more recent years, folic acid fortification of wheat has become widespread in the Americas, a strategy adopted by Canada and the USA and about 20 Latin American countries (WHO and FAO, 2006a).

In Brazil, in 2001, the Ministry of Health made the addition of iron mandatory (30% RNI or 4.2 mg per 100 g) and folic acid (70% RNI or 150 mg) to milled wheat and corn flour. Federal law now dictates mandatory fortification of iron instead of voluntary fortification by the grain industry. This measure has as its core objective of increasing the accessibility of milled cereal grains fortified with iron and folic acid, consumed by the Brazilian population to reduce the prevalence of iron deficiency and neural tube defects in Brazil (ANVISA, 2009). However, a time series analysis was done in the city of Pelotas in the south region of Brazil with three assessments every 12 months. In May 2004, before the flour fortification, levels of Hb were measured in a probabilistic sample of 453 children. After 12 and 24 months, 923 and 863 samples of children respectively, were analyzed. Iron fortification in flour was ineffective to increase the average levels of Hb in children from 0 to 71 months of age, after one and two years of intake. It can be partially explained not only by the insufficient flour intake by children of that range of age, but also by the low added-iron bioavailability or due to habitual intake of food with high levels of iron absorption inhibitors (Assunção *et al.*, 2007).

Food fortification can take several forms. It is possible to fortify foods that are widely consumed by the general population (mass fortification), to fortify foods designed for specific population subgroups, such as complementary foods for young children or rations for displaced populations (targeted fortification) and/or to allow food manufacturers to voluntarily fortify foods available in the market place (market-driven fortification) (WHO and FAO, 2006a).

The fortification of foods with iron is a preferred strategy advocated by the WHO. Iron added to foods has been shown to be the most efficient option to control iron-deficiency, and studies have shown improvements over a period of one to three months in people suffering from this deficiency (Nilson and Piza, 1998). Once foods are enriched with micronutrients, such as iron, large, at-risk populations will be reached over long periods without the need of effective individual cooperation (Tuma *et al.*, 2003; WHO, 1989). Therefore, food fortification is considered highly effective and flexible, is socially acceptable and furthermore, it does not interfere with the population's dietary habits. In addition, the risk of side effects and toxicity are minimal due to reduced doses of micronutrients added to foods (Tuma *et al.*, 2003).

Food fortification is a public health measure, and in order to be successful, several considerations should be kept in mind. First, the targeted population must consume the food vehicle of choice regularly and in large scale. In addition, the selected food vehicle should be evaluated for potent absorption inhibitors, and if the added iron compound will have an impact on the iron status of the consumer. Secondly, it is important that the selected iron compound does not cause

unacceptable changes in color and flavor when added to foods. Additionally, the food vehicle should be sufficiently stable during long periods of storage and during cooking in order to guarantee that true food consumption may be quantitatively capable of contributing significantly to the nutritional requirements of the population. Finally, the food vehicle must be centrally produced and proper technology is available for industrial-scale fortification (Cardoso and Penteado, 1994).

The direct costs of food fortification are extremely low when compared to the social costs of deficiency. It costs less than one dollar per year to protect people against vitamin A, iron and iodine deficiency (Pan American Health Organization, 2002).

10.3 Fortification of water

The addition of iron to potable drinking water is one alternative in the control and prevention of iron deficiency and anemia. This rather simple method can reach a large part of the developing country populations at each level of the social-economic stratum by the use of drinking water on a daily basis. Drinking water, other than used for drinking, is commonly used for preparation of foods, which may contribute even more towards increasing iron ingestion (Ferreira *et al.*, 1991).

It has to be clear, that a fortified food for anemia prevention to be effective in developing countries, should use carriers that are locally available and legally implemented in the community. Certainly one such alternative is the utilization of drinking water as an iron carrier. Water by itself is an essential liquid nutrient and other nutrients, like iron and other minerals and vitamins, could be dissolved in it (WHO, 2005). Water is available and used daily everywhere by everyone from infants at birth to old age, which does not happen with various other nutrient caarying foods. An important characteristic of water as a carrier is that it is liquid, proper to be given to infants, children, and sick people. The bioavailability of nutrients carried in water increases with their solubility. Fortification of drinking water with iron is a simple process, and solutions may be prepared locally starting with a concentrate water iron sulfate solution at a drug medicine store or similar set-up, always available locally, even in small towns. A concentrated iron solution is bottled in a 1 l pot and sent to the day-care centers where a diluted solution is offered to the children. At the daycare, a calculated amount of the concentrated iron solution is diluted to large drinking water bottles of 20 l (Dutra-de-Oliveira *et al.*, 2011).

Studies about water as a vehicle for food fortification with iron began in 1995 with an experimental study aiming to show the effect of adding different iron salts to drinking water and checking their effects on color, turbidity, and taste, through physical and chemical tests. Ferric ammonium citrate, ferric chloride, ferric gluconate, ferric hydroxide, ferric sulfate, and ferric nitrate, diluted in water, were tested. Iron citrate, chloride, gluconate, and ferrous sulfate produced small color changes at 1 and 5 mg/l concentrations, when measured initially and after seven days. Tests with iron sodium EDTA added to the water solution showed practically no color change and no metallic taste; the solution remained clear and transparent. The presence of chloride in iron

fortified water increased color and turbidity, though it had no effect on NaFeEDTA solution. The study also found that iron-fortified water turbidity decreases with the addition of ascorbic acid or citric acid to the solution. Water solutions of Fe gluconate, citrate, and sulfate had less color and turbidity than those of polimaltosed $FeOH_3$ and Fe^{3+} nitrate. An anemic weanling rat test was carried out where a group of rats did not get iron in their drinking water and the other group received water with ferrous sulfate, NaFeEDTA, iron-glycine chelate, and complex ferric orthophosphate. The control animals became anemic after 35 days, 9.1 g/dl of Hb, and the ones receiving ferrous sulfate, NaFeEdta, and Fe glycine chelate 13.0-13.9 g of Hb. The animals who consumed iron Fe orthophosphate had 10.6 g Hb/dl. Weight gain, Hb, hematocrit, transferring saturation, iron Hb, and relative iron biological value were checked in all animals. NaFeEDTA and iron aminochelate produced similar results as ferrous sulfate. Orthophosphate iron had lower biological value (Dutra-de-Oliveira *et al.*, 1995). From these results ferrous sulfate ($FeSO_4 \times 7H_2O$) was shown to be the best option, considering high availability and low price.

Some clinical work has been carried out in preschool children with iron-fortified drinking water in Brazil. These studies differ in some aspects such as the fortification compounds used (ferrous sulfate only or ferrous sulfate with L-ascorbic acid), the concentration (5-20 mg Fe/l) of those compounds and the duration of the treatment from four to eight months (Table 10.1).

Dutra-de-Oliveira *et al.* evaluated 31 preschool children aged 2-6 years enrolled in day-care facilities in Ribeirao Preto, Sao Paulo, Brazil. During eight months, children consumed iron-fortified drinking water (20 mg iron per l), which resulted in a significant decrease in the prevalence of anemia. At baseline, anemia prevalence was diagnosed in 58% of subjects; at four months, 16% continued anemic, and finally, at eight months post-study intervention, anemia was significantly reduced to only 3% of subjects. Mean Hb levels at baseline (10.6±1.1 g/dl) increased significantly to 12.1±1.4 g/dl at four months and 13±1.1 g/dl at the end of study (Dutra-de-Oliveira *et al.*, 1994).

The effectiveness of iron-fortified drinking water was also analyzed in low-income families. Participants were families of small children with anemia who were previously seen at our University Hospital in the City of Ribeirão Preto and having borderline blood iron deficiency anemia parameters. 21 families of low socioeconomic status including 88 subjects participated in this study and were divided into two groups. 12 families, 22 fathers and/or mothers, and 22 children less than six years old with Hb levels ≤11 g/dl participated in the study. Daily intakes of selected nutrients were measured. A solution of water iron plus ascorbic acid in small bottles was given to a group of 12 families' mothers to be added to their cooking pots (concentration 10 mg of Fe and 60 mg ascorbic acid per l). Nine other families (18 fathers and mothers and 26 similar children) were supplied with the same type of water, but without added iron or ascorbic acid, during four months. Blood samples were collected at the beginning and at the end of the trial to measure Hb and ferritin levels at the end in both children and adults. Iron-fortified drinking water increased Hb (children: 10.9±1.1 to 11.7±1.1 g/dl, $P<0.01$; adults: 12.9±1.7 to 13.7±1.7 g/dl, $P<0.01$) and ferritin (children 27.6±21.6 to 33.8±22.1 ng/dl; adults: 74.8±41.3 to 106.2±93.9 ng/dl, $P<0.05$). No significant changes in Hb and ferritin were found in the placebo group after

Table 10.1. Clinical work carried out in preschool children with iron fortified drink water in Brazil.

Reference	Age group (years)	Number of participants	Place	Fortification compounds	Indicator of iron deficiency, hemoglobin (g/dl)		Duration (months)
					Before	After	
Dutra-de-Oliveira *et al.* (1994)	children (2-6)	31	daycare center of Ribeirão Preto, Brazil	ferrous sulfate (20 mg Fe/l)	10.6±1.1	13.0±1.1	8
Dutra-de-Oliveira *et al.* (1996)	adults and children (1-6)	88	homes of Ribeirão Preto, Brazil	ferrous sulfate (10 mg/l) and L-ascorbic acid (100 mg/l)	children: 10.9±1.1, adults: 12.9±1.7	children: 11.7±1.1, adults: 13.7±1.7	4
Almeida *et al.* (2005)	children (1-6)	150	daycare center of Monte Alto, Brazil	ferrous sulfate (10 mg Fe/l)	11.3±1.3	11.5±1.3	6
Beinner *et al.* (2005)	children (0.5-6)	160	daycare center of Diamantin, Brazil	ferrous sulfate (12 mg/l) and L-ascorbic acid (90 mg/l)	11.8±1.3	12.4±0.93	8
Rocha *et al.* (2011)	children (0.5-6)	321	daycare center Belo Horizonte, Brazil	ferrous sulfate (5 mg/l) and L-ascorbic acid (100 mg/l)	11.8±1.3	12.9±1.4	5

four months. The authors concluded from this study that iron plus ascorbic acid fortification can be added for home food cooking and is effective to increase blood Hb and ferritin in adults and children. It can be a simple and effective way to supply iron to low socioeconomic families where the iron intake may be found to be low (Dutra-de-Oliveira *et al.*, 1996).

In order to clarify the role of ascorbic acid as a vehicle to enhance iron absorption a study on 150 children was conducted, who were frequenting six day-care centers, in the city of Monte Alto, southeast of Brazil. They were divided into two groups by drawing lots: iron-C group (three day-care centers, n=74) which used water fortified with 10 mg elemental iron and 100 mg ascorbic acid per l; and a comparison group (three day-care centers, n=76) which used water containing only 100 mg ascorbic acid per l. Anthropometric measurements and determinations of capillary Hb were performed at the beginning of the study and after six months of intervention. A fall in the prevalence of anemia and an increase in mean Hb levels associated with height gain were observed in both groups. This study showed that an increased iron supply by fortification of drinking water is a quite simple strategy of previously demonstrated effectiveness. Also, this study

demonstrated that for populations receiving an abundant supply of non-heme iron it is possible to control anemia in a quite simple, safe and inexpensive manner by adding only ascorbic acid to drinking water (De Almeida *et al.*, 2005).

Another study also using iron-fortified drinking water with vitamin C was conducted with 160 preschool children from eight municipal daycare facilities who benefited from daily consumption of iron (12 g element iron per l) plus ascorbic acid (90 mg/l) prepared in 20-l plastic water jugs. Mean Hb at baseline and after eight months of intervention increased significantly from 11.8±1.3 to 12.4±0.93 g/dl (P<0.01), respectively. The prevalence of iron deficiency determined by Hb levels decreased from 43.2 to 21% at eight months post-intervention. Fundamentally important to the success of this study was education of the targeted population, which resulted in behavior change and a greater awareness of the importance of combating iron deficiency and anemia by the use of iron-fortified drinking water (Beinner *et al.*, 2005).

In another study performed in the city of Belo Horizonte, Minas Gerais State of Brazil, the iron fortified water was not used only for drinking but also in food preparation. Thus, all meals offered in the daycare centers were made with the use of fortified water. In addition, the consumption of fortified water was ad libitum, from a single source. This extra amount of iron was incorporated into the daily diet of children. The amounts of iron and vitamin C used were based on previous studies by (Almeida *et al.*, 2005; Beinner *et al.*, 2005; Dutra de Oliveira *et al.*, 1994). However, the concentration used in our study − 5 mg of elemental iron and 50 mg of ascorbic acid per liters of water − was lower than those used in the previous studies. A total of 318 children aged 6 to 74 months were evaluated in day-care centers in Belo Horizonte. There was an overall reduction of 73% in the prevalence of anemia after five months, from 29.3% before fortification to 7.9% after fortification, with a significant increase in Hb levels, from 11.84±1.30 to 12.98±1.35 g/dl (P<0,001), an increase of 9.62%. Reductions in the prevalence rates of stunting and underweight were also observed. Consumption of water fortified with doses of iron and vitamin C lower than those used in previous studies resulted in improvement in Hb levels and, consequently, reduction of the prevalence of anemia in children from attending day-care centers. This study also showed an improved cost-benefit ratio compared with previous studies, since a lower concentration of iron and vitamin C had the same level of efficacy at a lower cost, with a mean cost of fortification of approximately 0.85 US$ per child per month. Thus, these data point to a viable strategy for the prevention and control of anemia in children attending day-care centers (Rocha *et al.*, 2011).

The use of drinking water as a vehicle for the control and prevention of iron deficiency and anemia is an effective and efficient model, which can be used in targeting preschool children enrolled at daycare facilities, and/or at the household level, which will include all family members. Consumption of drinking water fortified with iron can contribute to increasing iron-intake to meet minimal RNI allowance of bioavailable iron acceptable to preschool children aged 6 to 59 months of age. Other than adding iron-concentrate to the appropriate number of liters of drinking water, it is easy to distribute and can be easily monitored (Rocha *et al.*, 2011).

10.4 Conclusion

Iron-deficiency anemia is a critical nutrition problem in childhood due to its high prevalence and its consequences, mainly in underdeveloped and developing countries. There is already consent that individual measures are not very effective to combat this deficiency in regions where the prevalence is high. Hence, a relevant way to prevent iron anemia is to increase the intake of iron. Thus, iron fortification of their usual food is considered an excellent strategy.

The original studies performed in Brazil showed that the strategy of iron fortification of drinking water for the prevention of iron anemia was effective, as drinking water was available everywhere and consumed daily.

Iron salts are low-priced, water soluble, effective, easily and locally prepared. They should be known and accepted as a rational, practical and professional preventive solution for iron-deficiency anemia, which is still a major problem of developing and underdeveloped countries in the world.

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11. Intake and dietary sources of heme and non-heme iron in children and adolescents: recommendations for preventing iron deficiency

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Abstract

Iron is present in a range of proteins in the cells of the human body and has several vital functions (e.g. transporter of oxygen in blood, facilitator of oxygen use and storage in muscles, part of iron-containing enzymes). Globally, iron deficiency anemia affects 24.8% of the world population, with the highest prevalence in preschool-age children (47.4%). In premature and low-birth weight infants, an extra iron supply is needed. In full-term infants, iron requirements increase after age 4-6 months from 0.27 to 11 mg/d during the remaining part of the first year. In preschool children the iron requirements are 7 mg/d and increase in primary school children to 10 mg/d. Iron requirements increase further during adolescence because of rapid growth. The availability of iron for absorption is determined by its chemical form (heme vs. non-heme) and the presence of enhancers and inhibitors consumed in the meal. The absorption and bioavailability of heme iron (available in animal products) is higher than for non-heme iron. Red meat is the highest heme iron source, while cereals, pulses, vegetables are considered important non-heme iron sources. Methodological issues related to iron intake assessments and to food composition data, iron supplements and fortification should be considered when interpreting iron intake data. The primary goals of dietary modification to avoid iron deficiency in children and adolescents are to increase the intake of iron-containing foods, and encourage a meal pattern with more enhancers, and fewer inhibitors of iron absorption.

Keywords: anemia, iron requirements, iron supplementation

Key facts

- Iron is present in a range of proteins in the cells of the human body and has several vital functions.
- Iron deficiency is defined as a condition in which there are signs of a compromised supply of iron to tissues and iron stores are exhausted.
- Globally, iron deficiency anemia affects between 1.5 and 1.7 billion people, which is 24.8% of the world population.
- There are two types of dietary iron: heme iron, found in animal flesh foods; and non-heme iron, found in legumes, flesh foods, and plants.
- Non-heme iron is less ef ciently absorbed in the body than heme iron and strongly affected by other foods ingested at the same meal.

Summary points

- The highest prevalence of iron deficiency anemia worldwide is found in preschool-age children and is higher among children living at or below the poverty level.
- Long-term iron intakes that are not suf cient to meet requirements may result in impaired work capacity in all age groups, and impaired cognitive and psychomotor development among children.
- Although non-heme iron is not absorbed as ef ciently as heme iron, it is an important dietary source because it comprises about 85% of total iron in a Western diet.
- In developed countries, breads and cereals are major contributors of total dietary iron intake in toddlers, followed by fruits and vegetables and meat.
- The main source of total iron and heme dietary iron in teenage boys and girls is meat products, while the main source of non-heme iron is bread.
- Age, sex, body mass index, vegetarianism and physical activity are factors associated with dietary iron intake in European adolescents.
- Consumption of iron-fortified foods and avoidance of tea or coffee with meals are effective alterations to enhance iron absorption in vegetarian children and adolescents or those with iron deficiency.

Abbreviations

AI	Adequate intake
DRI	Dietary reference intake
EAR	Estimated average requirement
IDA	Iron deficiency anemia
RDA	Recommended dietary allowance

11.1 Introduction

This chapter describes the physiological processes involving iron and the prevalence and consequences of iron deficiency and iron overload in children and adolescents. In addition, the absorption and bioavailability of heme and non-heme iron, as well as iron losses are discussed in the context of iron requirements. The iron intake recommendations in children and adolescents are presented and compared with reported intakes while highlighting the most important heme and non-heme iron sources and factors affecting dietary iron intake in children and adolescents.

Furthermore, methodological issues related to the assessment of dietary iron intake among children and adolescents and to food composition data, iron supplements and fortification are discussed. Lastly, some general recommendations to avoid iron deficiency in children and adolescents are given.

11.2 Physiological processes involving iron

Iron is present in a range of proteins in the cells of the human body and has several vital functions. In the form of hemoglobin, it serves as a transporter of oxygen in the blood. Each single hemoglobin molecule contains two globin chains and is composed of four units, each comprising one protein chain and one iron-containing heme group which allows hemoglobin to be loaded with oxygen in the lungs and partially unloaded in the tissues (FAO/WHO, 2001). As myoglobin, iron also serves as a facilitator of oxygen use and storage in the muscles. Myoglobin is similar in structure to hemoglobin but has only one heme unit and one globin chain. There are several iron-containing enzymes in the human body. For example, the cytochromes have one heme group and one globin protein chain and carry electrons within the cells, specifically in the mitochondria. Other functions of the iron-containing enzymes include the synthesis of steroid hormones and bile acids (FAO/WHO, 2001).

11.3 Iron deficiency in children and adolescents

Iron deficiency is defined as a condition in which there are signs of a compromised supply of iron to tissues and iron stores are exhausted. The more severe stages of iron deficiency are associated with anemia.

When iron-deficient erythropoiesis occurs, hemoglobin concentrations are reduced but still above the cut off used to define anemia (Table 11.1). Nevertheless, the prevalence of IDA in a population may be a statistical rather than a physiological concept, as IDA is considered to be present when hemoglobin levels are below two standard deviations (-2D) of the distribution mean for hemoglobin in an otherwise normal population of the same gender and age who are living at the same altitude (WHO, 2001). Because anemia is the most common indicator used to screen for iron deficiency, the terms anemia, iron deficiency, and IDA are sometimes used interchangeably. This practice should be avoided because IDA represents the extreme lower end of the distribution of iron deficiency so there are iron deficiency states in which, although anemia is absent, tissues may still be functionally impaired. Furthermore, iron deficiency is not the only cause of anemia so anemia is not synonymous with IDA.

11.3.1 Prevalence of iron deficiency

The WHO defines anemia as a condition in which the number of red blood cells, or their oxygen-carrying capacity, is insufficient to meet physiological needs, which vary by age, sex, altitude, smoking, and pregnancy status. IDA is the most common form of anemia. Globally, IDA affects between 1.5 and 1.7 billion people, which is 24.8% of the world population. Even though the highest prevalence is in preschool-age children (47.4%), the population group with the greatest number of individuals affected is women of childbearing age (446-491 million women) (WHO, 2008).

Rates of IDA vary by race and other sociodemographic factors. For preschool-aged children, Africa has the highest prevalence of anemia (67.6%), but the greatest number of preschool-aged

Table 11.1. Hemoglobin and hematocrit cutoffs used to define anemia at sea level (adapted from WHO, 2001).

Age or sex group	Hemoglobin below (g/dl)	Hematocrit below (%)
Children 6 months to 5 years	11.0	33
Children 5-11 years	11.5	34
Children 12-13 years	12.0	36
Nonpregnant women	12.0	36
Pregnant women	11.0	33
Men	13.0	39

children affected are in Asia (115.3 million), accounting for 58% of the global anemia burden (McLean *et al.*, 2009). When countries are considered by category of development, the prevalence of iron deficiency is higher among children living at or below the poverty level than among those living above the poverty level (McLean *et al.*, 2009).

Both ethnicity and food security have been shown to be associated with iron deficiency and IDA. In the USA, the prevalence of iron deficiency is higher among black or Mexican-American children than among white children (CDC, 1998). In the case of pre-schoolers, 6% of white and black toddlers are iron deficient compared with 12% of Hispanic toddlers. Food insecurity is also associated with IDA in USA children and adolescents (Brotanek *et al.*, 2007; Eicher-Miller *et al.*, 2009), with iron deficiency more common in food-insecure households. Children fed on cow's milk or with prolonged breast feeding have a much higher prevalence of iron deficiency. Premature infants need extra iron supplements because the amount of body iron at birth is low due to insufficient accumulation during gestation (Marx, 1997).

Also, in many tropical countries, infestations with hookworms, ascaris and schistosomiasis lead to intestinal blood losses (FAO/WHO, 2001). Acute and chronic infections, including malaria, tuberculosis, and HIV, as well as some cancers, can also lower blood hemoglobin concentrations (WHO, 2008).

11.3.2 Consequences of iron deficiency

Long-term iron intakes that are not sufficient to meet requirements may lead to IDA, resulting in impaired work capacity in all age groups, and impaired cognitive and psychomotor development among children (Jauregui-Lobera, 2014). However, iron deficiency is a concern even before anemia develops, as individuals with iron depletion have no iron stores to mobilize if iron is needed in response to a physiological challenge, such as rapid growth.

There is current focus on the possibility that there may also be a relationship between non-anemic iron deficiency and impaired physical endurance and work capacity (Brownlie *et al.*, 2004), and various brain functions, such as mood (Vahdat Shariatpanaahi *et al.*, 2007) and learning (Fretham *et al.*, 2010). However, there is considerable controversy about whether these deficits, particularly in brain function, are present in the absence of anemia (Richardson *et al.*, 2015).

11.4 Iron toxicity

Conversely, up to one million persons in the USA may be affected by iron overload due to hereditary hemochromatosis (CDC, 1998). The European countries with the highest prevalence include Ireland, France and Denmark (Adams, 2005). In these populations, hemochromatosis is most commonly associated with homozygosity for the C282Y mutation of the HFE gene, a common genetic mutation occurring in 0.3 to 0.5% of persons of northern European descent (Adams *et al.*, 2005). Hemochromatosis is characterized by excessive iron absorption, excess tissue

iron stores and potential tissue injury. If hemochromatosis is not detected and treated, people can develop signs of iron toxicity such as cirrhosis of the liver, diabetes and cardiomyopathy (NIH, 2015). Hereditary hemochromatosis is, however, almost entirely a disease of middle and older age and is effectively treated, once diagnosed, by periodic phlebotomy. There is a range of other pathological iron overload states that are seen in children and adolescents but these are rare (Adams, 2005).

11.5 Mechanisms of iron regulation

11.5.1 Heme and non-heme iron absorption and bioavailability

There are two types of dietary iron with respect to the mechanism of absorption: heme iron, found in animal flesh foods such as meat and constituting up to 15% of the dietary iron; and non-heme iron, found in legumes, flesh foods, and plants, and constituting the remainder. In the case of vegetarian diets, all dietary iron will be non-heme iron.

Non-heme iron is present in foods as either ferric (Fe^{3+}) or ferrous (Fe^{2+}) salts (Zijp *et al.*, 2000). Heme iron is found primarily in hemoglobin and myoglobin (Vandevijvere *et al.*, 2013), and consists of a protoporphyrin IX molecule containing a central Fe^{2+} atom liganded to the four nitrogen atoms of the porphyrin ring (Figure 11.1). There are two further ligand binding sites which are free to interact with other molecules. The porphyrin complex of heme iron allows the molecule to prevent oxidation to Fe^{3+}, its insoluble form (Voet *et al.*, 2008).

Heme and non-heme iron absorption are affected differently, not only by dietary factors, but also by the iron status of the individual. The percentage of iron absorbed can vary from <1% to >50% (Hallberg, 1981). The main factor influencing iron absorption is the amount of iron stored in the

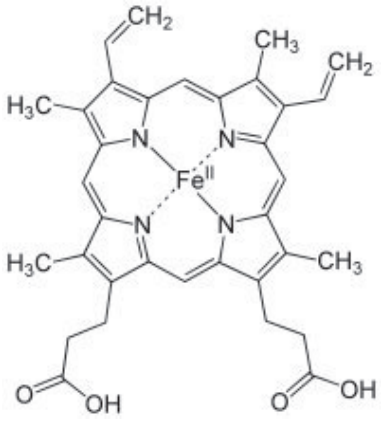

Figure 11.1. Molecule of heme iron.

body. Low iron stores in an individual increase the amount of dietary iron absorbed. Conversely, positive iron balance reduces absorption (Hurrell and Egli, 2010).

Regulation of iron balance occurs mainly in the gastrointestinal tract through absorption (there is no mechanism for controlled excretion of iron). When the absorptive mechanism is operating normally, the body maintains functional iron and tends to establish iron stores. The availability of iron for absorption is determined by its chemical form (heme vs. non-heme) and the presence of enhancers and inhibitors consumed in the meal (Hallberg and Hulthen, 2002).

Ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) in the intestinal lumen by a ferrireductase (Figure 11.2). Fe^{2+} is transported into the enterocyte by divalent metal ion transporter-1 (DMT-

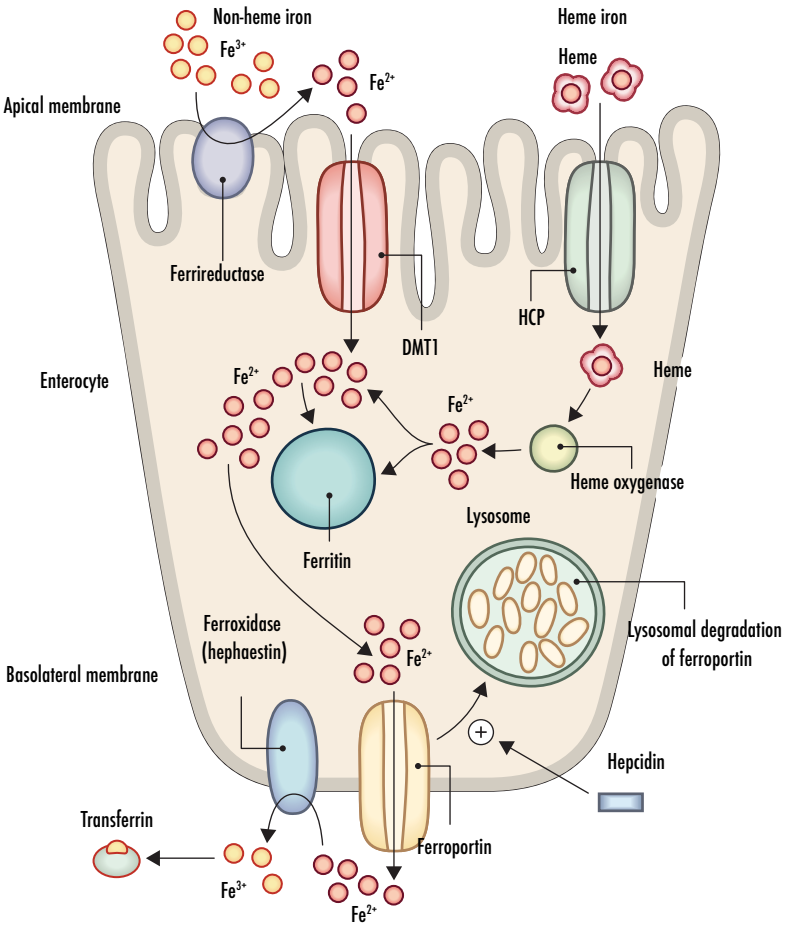

Figure 11.2. Iron absorption (modified after Rizvi and Schoen, 2011).

1). It is not known how dietary heme iron is transported across into the enterocyte; the specific heme carrier protein has not yet been identified.

Inside the enterocyte, the enzyme heme oxygenase releases ferrous iron from the protoporphyrin in heme. The released iron enters the same pool as non-heme iron. A portion of this pool is stored as ferritin inside the enterocyte, which is later lost with sloughing of the intestinal mucosa. The remainder of the Fe^{2+} is transported across the basolateral membrane of the enterocyte by ferroportin. The transported Fe^{2+} is oxidized back to the Fe^{3+} form by ferroxidase. Fe^{3+} is then incorporated into transferrin in the blood to be transported to other tissues (Rizvi and Schoen, 2011).

Heme iron is at least twice as well absorbed as non-heme iron (i.e. 25-35% cf 5-15%) (Hallberg, 1981), and dietary constituents have little effect on heme iron making it more readily available for absorption. The bioavailability of non-heme iron is strongly affected by other foods ingested at the same meal (Table 11.2). Vegetarian diets, by definition, do not contain heme iron so are particularly vulnerable to the effects of inhibitors of iron absorption. However, iron bioavailability can be increased by choosing meals that include enhancers of iron absorption (CDC, 1998).

Fe^{3+} is insoluble at pH >3. To avoid precipitation, it is therefore solubilized and chelated by dietary components in the stomach so it can be absorbed in the duodenum. The chelators can either enhance absorption by forming low-molecular-weight chelates which can easily be solubilised, or reduce non-heme bioavailability by forming high-molecular-weight chelates (Table 11.2) (MacPhail, 2007).

Phytates strongly inhibit non-heme iron absorption and even small amounts have a marked effect (Gillooly *et al.*, 1983; Hallberg *et al.*, 1989). Bran and oats both contain high levels of phytates, therefore whole wheat flour has a much higher content of phytates than white flour. However, because the less highly phosphorylated forms of phytate do not appear to inhibit iron absorption (Brune *et al.*, 1992), a number of food processing methods can substantially reduce the inhibitory

Table 11.2. Enhancers and inhibitors of iron absorption (adapted from MacPhail, 2007).

Iron absorption enhancers	Iron absorption inhibitors
Ascorbic acid	Phytates
Meat	Polyphenols
Fish	Calcium
Poultry	Vegetable proteins (e.g. soy protein)
Other organic acids (e.g. citric acid)	Phosphates
	Oxalic acid
	High doses of zinc, magnesium or copper

effect of phytate on iron absorption, including: fermentation (e.g. yeast fermentation in bread making), germination, and high pressure heating (e.g. canning).

Almost all plants contain phenolic compounds as part of their defense system against insects, animals, and humans. Some types of phenolic compounds, like galloyl groups, seem to inhibit iron absorption. Green leafy vegetables, herbs and spices are rich in galloyl groups. Tea, coffee and cocoa therefore also contribute to the inhibition of iron absorption because of their high content of other iron-binding polyphenols (Disler *et al.*, 1975; Hallberg and Rossander, 1982), although cocoa is also a source of iron (Yokoi *et al.*, 2009).

Calcium interferes with the absorption of both heme and non-heme iron (Hallberg *et al.*, 1991), however it does not appear to impact on iron status (Minihane and Fairweather-Tait, 1998). Moreover, calcium is an essential nutrient, therefore, it is recommended to increase iron intake instead of reducing calcium intake (Hallberg *et al.*, 1991) or consume the sources of both minerals separately.

Ascorbic acid is a potent enhancer of non-heme iron absorption and it has been proposed that each meal should preferably contain at least 25 mg of ascorbic acid and possibly more if the meal is rich in inhibitors for iron absorption (FAO/WHO, 2001). Other organic acids, such as citric acid, have also been found to enhance the absorption of non-heme iron (Gillooly *et al.*, 1983).

Meat, fish, poultry and seafood also promote the absorption of non-heme iron, although the mechanism of this effect is still controversial (Armah *et al.*, 2008; FAO/WHO, 2001; Huh *et al.*, 2004). Epidemiological studies have found that higher meat intake is associated with a lower prevalence of iron deficiency (Moshe *et al.*, 2013).

A number of algorithms have been developed in an attempt to use dietary intake data to estimate iron absorption (Heath and Fairweather-Tait, 2002). The more recent algorithms take into account many of the enhancers and inhibitors of iron absorption (Hallberg and Hulthen, 2000). However, the algorithms require data on the phytate and tannin content of foods, and there are as yet no extensive databases of these available (Heath and Fairweather-Tait, 2002). Moreover, although they may be useful for comparing the bioavailability of different diets, they do not provide an accurate absolute measure of absorbable iron (Beard *et al.*, 2007).

11.5.2 Iron losses

Iron is lost via sloughing off of intestinal mucosal cells, and via other blood losses, including menstruation in women. In adult men, approximately 14 μg/kg body weight/d (i.e. approximately 0.9 mg/d) of iron is lost daily and the range of individual variation has been estimated to be ±15% (FAO/WHO, 2001).

After menarche, the additional iron losses as a result of menstruation in teenage girls are approximately the same as those in adult women, about 0.56 mg/d (FAO/WHO, 2001). It is estimated that children from 1 to 10 years of age lose between 0.19-0.39 mg of iron/d (FAO/WHO, 2001)

11.6 Iron requirements and intake recommendations in children and adolescents

Iron intake recommendations are based on requirements, adjusted to take into account the amount of iron that would need to be consumed for these requirements to be met (because of the relatively low bioavailability of iron). Intake recommendations for iron and other nutrients are provided in the DRIs developed by the Food and Nutrition Board at the Institute of Medicine of the National Academy of Sciences (IOM, 2001). These values, which vary by age and sex, include:

- EAR: the daily intake level of dietary iron sufficient to meet the needs of 50% of healthy individuals in a particular age and sex group.
- RDA: the amount that will meet the daily requirement of almost all individuals, i.e. 97-98%.
- AI: established when evidence is insufficient to develop a RDA. Intake above this level is assumed to indicate nutritional adequacy.

The distribution of iron requirements is skewed to the right which necessitates other approaches than the classical balance study method to determine the average requirements for the various age and gender groups. Such alternative approaches, for instance factorial modelling, propose daily physiological requirements for absorbed iron based on estimates of basal losses (e.g. through feces, urine, sweat and exfoliation of skin) and, where relevant, menstrual losses and needs for iron accretion in periods of growth (IOM, 2001).

Table 11.3 lists the current iron DRIs for non-vegetarians. The EARs and RDAs for vegetarians are 1.8 times higher than for people who eat meat due to the lower iron bioavailability of their diets because they do not consume heme iron or the iron absorption enhancer found in meat, fish and poultry.

In the premature and low-birth weight infant, an extra supply of iron is needed. In the full-term infant, iron requirements will increase after age 4-6 months from 0.27 mg/d to about 11 mg/d during the remaining part of the first year, and this is reflected in a substantial increase in their recommended intake of iron (Table 11.3).

Iron requirements are also very high during adolescence because of rapid growth. There is marked inter-individual variation in growth rate depending on the age at onset of puberty. In boys, increased muscle development leads to increased demands for iron, while in girls, in addition to rapid growth, the onset of menstruation leads to iron losses.

Table 11.3. Dietary reference intakes for iron in non-vegetarian children and adolescents (mg/d) (IOM, 2001).[1]

	AI	EAR	RDA
Infants			
0 to 6 months	0.27		
6 to 12 months		6.9	11
Children			
1-3 years		3.0	7
4-8 years		4.1	10
Males			
9-13 years		5.9	8
14-18 years		7.7	11
Females			
9-13 years		5.7	8
14-18 years		7.9	15
Pregnancy			
14-18 years		23	27
Lactation			
14-18 years		7	10

[1] AI: adequate intake; EAR: estimated average requirement; RDA: recommended dietary allowance.

11.7 Heme and non-heme iron sources and intakes in children and adolescents

Heme iron is a component of hemoglobin and myoglobin and is therefore found only in animal tissue. While it has been estimated that red meat such as beef may have 50% or more of its iron as heme iron, considerably less heme iron is provided by a portion of chicken or fish (Kongkachuichai *et al.*, 2002).

The main dietary sources of non-heme iron include cereals, pulses, vegetables and meat. Although non-heme iron is not absorbed as efficiently as heme iron, it is an important dietary source because it comprises about 85% of total iron in the typical Western diet (Heath and Fairweather-Tait, 2002).

11.7.1 Children

Lambert *et al.* (2004) collected information on the dietary intake of European children and adolescents. Iron intake in children aged 2 to 3 years ranged from approximately 5 to 10 mg/d and in those aged 4 to 6 years from 6 to 13 mg/d. Among boys aged 7 to 10 years, the lowest intake of about 8.7 mg/d was observed in France and the highest in Finland and urban regions of

Estonia and Sweden. The greatest intake of iron by girls aged 7-14 years was reported in Russia (Lambert *et al.*, 2004). In the USA the average daily iron intake from foods is 11.5-13.7 mg/d in children aged 2-11 years (NIH, 2015).

Studies in developed countries have shown that breads and cereals are major contributors of total dietary iron intake (30-60%) in toddlers aged 12-24 months, followed by fruits and vegetables (15-26%) and meat and meat dinners (10-25%) (Huybrechts *et al.*, 2012; Soh *et al.*, 2002).

In toddlers, processed meats and chicken nuggets are the most commonly consumed meat items and meat, poultry and fish intakes range between 20-39 g/d (Fox *et al.*, 2006; Szymlek-Gay *et al.*, 2009).

Ready-to-eat cereal is the major contributor to iron intake among USA children aged 6-11 years (27%). Beef (8%) and yeast bread (13%) are also important contributors. Cakes, cookies, quick breads, donuts and pasta also are among the top five food sources of iron in USA children of either sex (Subar *et al.*, 1998).

11.7.2 Adolescents

In Europe, there were no clear differences in intakes between Southern and Northern countries. Adolescent girls aged 12.5-17.5 years have a lower mean total iron intake (11 mg/d) than boys (13.8 mg/d) and in addition, their ratio of non-heme/heme iron intake is higher compared to boys (Lambert *et al.*, 2004; Vandevijvere *et al.*, 2013). The highest intake of iron by girls aged 15 to 18 years (15.2 mg/d) was reported in Sweden (Lambert *et al.*, 2004), an intake similar to that reported in the USA where the average daily iron intake from foods in teens aged 12-19 years is 15.1 mg/d (NIH, 2015).

For boys, the top three contributors to heme iron intake were meat (76.7%), bread and rolls (3.8%) and soups and bouillon (3.8%), while for girls, these were meat (75.7%), fish products (4.4%) and bread and rolls (4.2%). For boys and girls, the top three contributors to non-heme iron intake were bread and rolls (14.1 and 13.8, respectively), meat (11.6 and 9.5%, respectively) and vegetables excluding potatoes (7.2 and 9.4%, respectively) (Vandevijvere *et al.*, 2013). In agreement with European adolescents, the main source of total iron and heme dietary iron in USA teenage boys and girls aged 12-19 years is meat products, while the main source of non-heme iron is bread (Subar *et al.*, 1998).

11.7.3 Factors affecting dietary iron intake

Age, sex, body mass index, vegetarianism and physical activity are factors demonstrated to be associated with dietary iron intake in European adolescents (Thane *et al.*, 2003; Vandevijvere *et al.*, 2013). In contrast, socio-economic status and the onset of puberty seem not to be associated with iron intake. Whilst adolescent boys, older adolescents, and obese adolescents have a more optimal iron intake, vegetarian adolescents report lower iron intakes than omnivores (Thane *et*

al., 2003). Interestingly, girls who watch more than 120 min/d of television appear to be prone to lower intakes of iron (Ramos *et al.*, 2013).

11.8 Methodological issues related to assessment of dietary iron intake

Several assessment methods can be used to investigate dietary intake of iron and its absorption modifiers. The 'gold standard method' is the diet record which requires the participant to estimate or weigh all food and drink consumed and record it for a number of days, ideally 12 days because of the large intra-individual variation (Heath and Fairweather-Tait, 2002). A less intensive method is the food frequency questionnaire.

Iron absorption modifiers are difficult to measure, because they act during the process of digestion and absorption of the iron-containing meal. To date, mean daily intakes of iron modifiers have been reported rather than intake by meal (FAO/WHO, 2001; Hallberg, 1981).

11.8.1 Food composition tables

Most food composition databases are compiled using a combination of direct and indirect methods, including both original analytical values, and values taken from the literature. This approach can be successful when key food sources of a nutrient are analyzed directly, and data for foods consumed in very limited amounts are taken from the literature.

The forms in which iron occurs, heme and non-heme iron, are important in relation to iron bioavailability, unfortunately, this information is only available in a small number of national food composition databases (Balder *et al.*, 2006).

Iron can be measured by atomic absorption spectrometry, however this technique only determines the total concentration of iron and provides no information on the oxidation state of iron, or the relative amounts of heme and non-heme forms. The method of Hornsey (1956) is the most commonly used method for determining heme iron in food samples. It involves extraction of heme using acidified acetone which is then measured on a spectrophotometer at 640 nm using a hematin standard curve (Hornsey, 1956). Inductively coupled plasma spectroscopy can also determine heme and non-heme fractions in both raw and cooked foods (Harrington *et al.*, 2001). Although it is possible to determine the heme iron concentration of both raw and cooked meats, it is not straightforward, and the determination of heme iron in cooked mixed dishes is still problematic. For this reason, heme iron concentration is often calculated as the difference between total iron and non-heme iron concentration.

11.8.2 Iron supplements and fortification

Globally, there has been an increase in the consumption of mineral and vitamin supplements, but these are rarely listed in food composition tables and databases (Charrondiere *et al.*, 2002). As a

result, it can be assumed that nutrient intake estimations are increasingly unrepresentative of total nutrient intake, particularly for nutrients such as iron and vitamin C. The absence of supplement information will impact on estimates of total and non-heme, but not heme iron intake.

Iron supplements may be used to prevent the development of iron deficiency, to prevent anemia by correcting iron deficiency before IDA is manifest, and to correct established IDA. Because of the widespread interest in iron intake and status, particularly in women, iron supplements are often taken even when they are not medically indicated or prescribed. WHO supports the use of iron supplements in population groups where prevalence of anemia is high (>40%) (Table 11.4).

Cases of iron overload have been reported due to excessive use of dietary supplements or medicines. In the USA, 43 children died by ingesting high doses (36-443 mg iron/kg body weight) of iron from supplements between 1983 and 2000. In Europe, the intake of iron supplements is much lower and cases of iron poisoning in children have not been reported (Anderson, 1994).

11.9 General recommendations to avoid iron deficiency in children and adolescents

The majority of cases of iron deficiency, like most nutritional deficiencies all over the world, occur as a consequence of poverty in developing countries (McLean *et al.*, 2009). Therefore,

Table 11.4. Dosage schedules for iron supplementation to prevent iron deficiency anemia (Stoltzus and Dreyfuss, 2011; WHO, 2001, 2011).

Age groups	Indications for supplementation	Dosage schedule	Duration
Low-birth-weight infants	Universal supplementation	2 mg iron/kg body weight/d	From 2 months of age up to 23 months of age
Children from 6 to 23 months of age	Where the diet does not include foods fortified with iron or where anemia prevalence is above 40%	2.5 mg iron/d	6-23 months of age
Children from 24 to 59 months of age	Where anemia prevalence is 20% or higher	25 mg iron/d	3 months of supplementation followed by 3 months of no supplementation after which the provision of supplements should restart
School-aged children from 5 to 12 years old	Where anemia prevalence is 20% or higher	45 mg iron/d	3 months of supplementation followed by 3 months of no supplementation after which the provision of supplements should restart

actions should be taken to reduce poverty, improve access to diversified diets, health services and sanitation, and promote better care and feeding practices. Even in developed countries, however, iron deficiency is present in some groups of the population.

The primary goals of dietary modification to avoid iron deficiency in children and adolescents are to increase the intake of iron-containing foods, and encourage a meal pattern with more enhancers, and fewer inhibitors of iron absorption.

According to the US National Institutes of Health, in order to consume and benefit from an adequate dietary iron intake, children and adolescents should:

- consume a variety of fruits, vegetables, and whole grains;
- include lean meats, poultry, seafood, beans and peas, eggs and nuts and seeds in their diet. Oysters and beef liver contain high amounts of iron, with chicken, tuna, and eggs also containing significant quantities.

Also, attention should be paid to children or adolescents raised in vegetarian or vegan cultures or households. Some examples of simple but effective alterations in meal patterns that can be applied to vegetarian children and adolescents, or those with iron deficiency, to enhance iron absorption might include:

- consumption of foods that are fortified with iron;
- avoidance of tea or coffee with meals;
- choosing breads that have been leavened with yeast.

Iron supplements should only be taken if these are prescribed by a doctor. The potential synergy between vitamins and minerals and other (perhaps still undiscovered) essential constituents of foods is one of the most compelling reasons for consuming a variety of whole foods rather than trying to achieve dietary recommendations through supplementation (Michaelsen, 2003). Furthermore, overuse of nutrient supplements could unbalance homeostasis as many minerals have similar molecular weight and hence compete for intestinal absorption (Celotti and Bignamini, 1999; Coppens *et al.*, 2006; Halsted, 2003). Therefore, excessive intakes of one micronutrient may adversely affect the absorption of other micronutrients.

Health organizations such as the American Dietetic Association and WHO stress that intake of a wide variety of foods is preferred over nutrient supplementation as a method for obtaining adequate vitamins and minerals (Marra and Boyar, 2009; Michaelsen *et al.*, 2003). However, it is important to emphasize that in some situations (e.g. children suffering from diseases causing nutrient inadequacies) dietary supplements are recommended in order to achieve or maintain adequate nutrient intakes. In these latter situations a medical doctor who diagnoses the child's nutritional status should prescribe the most optimal dietary supplement.

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Foods in blood diseases

12. Enteral nutrition with L-glutamine in sepsis patients

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Abstract

Glutamine is profoundly depleted in plasma and tissues during catabolic stress conditions, such as trauma, surgery, extensive burns, sepsis and inflammatory processes, and, as a nonessential amino acid, becomes a 'conditionally essential' amino acid in critically ill patients under stress conditions. The effect of enteral nutrition supplemented with L-glutamine in patients with sepsis showed that the use of glutamine, despite being supplied enterally, increased the percentage of lymphocytes, indicating that the amino acid was not retained by the enterocytes. The finding of an increased percentage and total number of lymphocytes indicates significant improvement of the immune system. Considering the observed benefits and the absence of adverse effects, enteral and parenteral diets enriched with L-glutamine or L-alanyl-glutamine are currently recommended for severe intensive care unit patients in order to modulate the immune system.

Keywords: nutraceutical, supplementation, metabolism, stressful conditions, immune system

Key facts

- Glutamine is a conditionally essential amino acid in stress conditions.
- Glutamine can be synthesized by the body in large enough amounts to satisfy nutritional needs under normal physiological conditions, in which case it is considered a non-essential amino acid.
- Glutamine is profoundly depleted in plasma and tissues during catabolic stress conditions, such as trauma, surgery, extensive burns, sepsis and inflammatory processes.
- Enteral nutrition supplemented with glutamine increases the percentage and count of lymphocytes, enhancing immune function.
- No adverse effects have been observed which may be attributed to glutamine in diets enriched with L-glutamine or L-alanyl-glutamine and glutamine-enriched enteral diets are well tolerated.

Summary points

- Glutamine is the most abundant amino acid in human circulation and plays an important role in normal cell metabolism and energy balance due to its capacity to be used as a source of energy. Despite large muscle stores, glutamine is profoundly depleted in plasma and tissues during stress conditions, such as trauma, surgery, extensive burns, sepsis and inflammatory processes.
- Glutamine is a conditionally essential amino acid in stress conditions.
- The role of glutamine has been recognized for over 40 years and several studies have shown that positive effects of enteral and parenteral supplementation with glutamine may be beneficial for critical patients.
- In sepsis glutamine depletion is a prognostic factor of clinical response.
- Enteral supplementation with glutamine is clinically well tolerated and free of side effects in intensive care patients.
- Infection complications can be reduced with glutamine supplementation.

Abbreviations

GSH	Glutathione
HSP	Heat shock protein
ICU	Intensive care unit
LDH	Lactate dehydrogenase
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid-reactive substances
SIRS	Systemic inflammatory response syndrome

12.1 Glutamine and metabolic functions

Glutamine is the most abundant amino acid in human circulation and plays important role in normal cell metabolism and energy balance due to its capacity to be used as a source of energy. However, glutamine has numerous valuable metabolic functions in nitrogen transport, maintenance of the cellular redox state and is a required component of GSH, the major intracellular antioxidant of the human body. As a nonessential amino acid, it becomes a 'conditionally essential' amino acid in critical ill patients under stress conditions? Research has suggested that glutamine availability may either be reduced by malnutrition or by failure to meet the demands due to critical illnesses.

Over the last two decades, evidence has supported the notion that glutamine, as an essential amino acid in stress conditions, has been receiving increasing attention from researchers and several studies have demonstrated its benefits.

Glutamine supplementation was extensively studied in both animal models and humans, but the most significant results have been observed when the body is under severe stress of sepsis, cancer, premature birth, and immunosuppression, where the glutamine in a nutraceutical dose has essential properties in lymphocytes proliferation and optimal function of neutrophils and macrophages.

Glutamine is primarily synthesized in skeletal muscle. It allows nitrogen to be transferred to the spleen, kidneys and immune system (Biolo *et al*., 2005). In humans, the plasma carries approximately 25% of the glutamine produced by the organism (Newsholme, 2001). Due to its ability to synthesize and release glutamine, especially during moments of increased demand from other organs and tissues, skeletal muscle plays a key metabolic role in the regulation of glutaminemia (Cruzat *et al*., 2009).

The discrepancy between the increase in glutamine utilization by the organism and the relative decrease in *de novo* glutamine synthesis in skeletal muscle leads to systemic glutamine deficiency and therefore to severe disease (Biolo *et al*., 2005).

Glutamine can be synthesized by the body in large enough amounts to satisfy nutritional needs under normal physiological conditions, in which case it is considered a non-essential amino acid. It is synthesized from glutamate and ammonium, catalyzed by glutamine synthetase in an ATP-dependent reaction (Moskovitz *et al*., 1994). However, despite being the most abundant amino acid in the human body, in stress states, while glutamine synthesis increases in the muscle in response to aggression, the metabolic demand exceeds the body's capacity to synthesize it.

Thus, despite large muscle stores, glutamine is profoundly depleted in plasma and tissues during catabolic stress conditions, such as trauma, surgery, extensive burns, sepsis and inflammatory processes (Wernerman, 2011). Once glutamine stores are depleted and endogenous production falls short of the demand, dietary glutamine supplementation becomes necessary. In this scenario, due to the combination of reduced food intake and upregulated metabolism, leading to depletion despite increased skeletal muscle synthesis, glutamine may be considered a conditionally essential amino acid (Arruda and Aguilar-Nascimento, 2004; Dupertius *et al*., 2009; Griffiths *et al*., 2002; Newsholme, 2001; Wernerman, 2011).

In a recent publication, Rajendram *et al*. (2015) organized the evidence on glutamine in clinical nutrition, thereby combining biochemistry, nutrition, as well as the therapeutic aspects of glutamine supplementation, in a practical way for health professionals.

Critical clinical illness leads to glutamine deficiency and intravenous glutamine supplementation decreased complications and infections associated with mortality in ICU setting (Fuentes-Orozco *et al*., 2004; Novak *et al*., 2002).

Observations suggest that intense catabolism (proteolysis) occurs in critically ill patients, and that in stressful conditions, the muscular ef ux of glutamine increases and intracellular sources of glutamine become depleted within several weeks. The levels of glutamine usually drop by 20% on the first day, and stay low throughout the critical period. Therefore, these patients require glutamine supplementation because glutamine levels in the plasma and cytosol levels drop significantly (Grintescu *et al*., 2014).

Clinical trials showed that glutamine-enriched parenteral nutrition in critically ill patients increased serum HSP levels and improves antioxidant defense (Abilés *et al*., 2008; Ziegler *et al*., 2005). Likewise, L-alanyl-glutamine enriched parenteral nutrition is known to augment glucose utilization in critically ill patients (Dechelotte *et al*., 2006) and to decrease insulin resistance in severe trauma patients (Bakalar *et al*., 2006). Thus, the glutamine supplementation potentially helps glucose control levels, probably by glycolysis stimulation.

12.2 Glutamine in sepsis

In sepsis, glutamine depletion is a prognostic factor of clinical response. The combination of reduced food intake and up regulated metabolism leads to glutamine depletion despite increased endogenous production in skeletal muscle (Coëffier *et al*., 2009).

Glutamine production may also be increased during sepsis, but stores are mainly consumed by the liver and immune cells, as compared to the intestine. In this condition, the liver becomes the main consumer of glutamine from skeletal muscle, which is the main source of glutamine endogenous production. In other words, glutamine supplementation allows restoring glutamine deficiency and reducing infectious complications (Coëffier *et al*., 2009).

The well-established, low-risk, low-cost intervention of glutamine therapy may protect organs against injury, attenuate inflammation, preserve metabolic function, and ultimately improve outcome in critically ill patients (Weitzel and Wischmeyer, 2010).

During severe infection, skeletal muscle, the most important repository of glutamine, releases twice the usual amount of glutamine, and endogenous glutamine biosynthesis increases significantly. Despite the increased activity of skeletal muscle glutamine synthetase, the intracellular glutamine pool becomes depleted, indicating that release rates exceed rates of synthesis. The fact that no increase is observed in the levels of circulating glutamine, suggests that uptake by other organs has increased concurrently (Karinch *et al*., 2001; Newsholme, 2001).

Under stress conditions, changes occur in the flow of glutamine among organs. In healthy individuals, glutamine production is mainly by skeletal muscle and the lungs, and is consumed by the intestine, immune cells and the liver. During sepsis, glutamine production increases, while most of the uptake occurs in immune cells and liver tissues, rather than in the intestine. In this scenario, the liver becomes the main organ of uptake of glutamine released by skeletal muscle, the principal glutamine supplier (Karinch *et al*., 2001).

High-dose glutamine supplementation is associated with improved post-surgical outcomes, including fewer infectious complications and shorter hospitalisation, with no adverse effect on mortality. In critical ill patients, glutamine supplementation has been associated with reductions in complications and mortality rates. However, supplementation yields the best results when administered parenterally (Novak *et al*., 2002).

12.3 Immune system

One of the most important action mechanisms of glutamine is in the immune system. Glutamine regulates the immune system and helps preserve the metabolic functions of tissues under stress.

Glutamine is a key substrate for activated immune cells, increases lymphocytic response to mitogen stimulation and alleviates bacteremia and endotoxemia, thereby helping prevent sepsis (Cynober, 2000). The high rate of glutamine utilization by lymphocytes and macrophages suggests that glutamine supply plays an important role in the maintenance of immune function (Calder and Yaqoob, 1999; Newsholme and Calder, 1997).

The activation of cells such as T-lymphocytes is essential for the maintenance of adequate immune response and contributes to preventing bacterial translocation (Burrin *et al*., 2000). It is important in cell growth and differentiation, inter-organ carbon transfer and as a preferential substrate for cells with rapid turnover, such as erythrocytes, and immune cells, such as macrophages and lymphocytes (Wilmore and Shabert, 1998).

Immune cells utilize large amounts of glutamine to sustain lymphocyte proliferation and cytokine production by lymphocytes and macrophages. In other words, the basic functions of these cells, as demonstrated *in vitro*, are dependent on glutamine supply (Calder and Yaqoob, 1999; Newsholme and Calder, 1997).

Normally and especially during stress, the digestive tract is the main organ of glutamine uptake and utilization since the intestinal mucosa contains immune and neuroendocrine cells in addition to numerous absorptive enterocytes. In response, the bowel perceives the nutritional and antigenic environment, acts in immunological screening and defense, and produces endocrine responses to the lumen environment (Burrin *et al*., 2000).

In short, the administration of glutamine or one of its precursors is beneficial to severely ill patients with inflammatory and immune response due to stress, trauma, sepsis or any condition that increases susceptibility to infection.

12.4 Oxidative stress

Oxidative stress reflects an imbalance between the production of ROS and the ability of the body's antioxidant defense mechanisms to detoxify reactive intermediates. ROS play an important role in the regulation of normal cell functions but, when produced in excess, can be harmful and lead to oxidative stress (Rosenfeldt *et al*., 2013).

Excessive ROS production can damage all cell components, including proteins, lipids and nucleic acids. The greater the oxidative stress, the more extensive the cell damage. Eventually, apoptosis (or even cell necrosis) may occur (Rosenfeldt *et al*., 2013).

Among the consequences of oxidative stress, lipid peroxidation is the most extensively investigated. It is measured by detection in plasma or urine of TBARS, a marker of lipid peroxidation. SIRS, a host response to infection and other forms of tissue injury, is often accompanied by oxidative

stress in one or more organic systems. In fact, Takeshi *et al.* (2003) observed that TBARS and lipid peroxidation increase in patients with SIRS (Takeshi *et al*., 2003).

Glutamine is a precursor of GSH which found in high concentrations in mammalian cells. GSH is considered the most potent antioxidant substance in the human body and is capable of neutralizing the action of ROS. It is the main component of the antioxidant defense system in cells and has a range of effects on the immune system, stimulating or inhibiting immune response in order to control inflammation (Fläring *et al*., 2003; Perricone *et al*., 2009).

Changes in GSH concentrations play an important role in several clinical conditions, especially in those involving inflammatory and immune responses mediated by oxidative stress reactions. In such situations, failure of the antioxidant defense system to maintain the rate of oxidative balance is usually due to the combination of weakened antioxidant defense and increased production of pro-oxidants (Perricone *et al*., 2009). Thus, glutamine depletion can lead to decreased GSH levels and may require replacement by exogenous glutamine supply (Bonet and Grau, 2007).

Hyperinflammation, cellular immune dysfunction, oxidative stress and mitochondrial dysfunction can be ameliorated by careful, targeted supplementation with key nutrients, such as glutamine, fat-soluble vitamins and antioxidants. In the REDOXS study, Heyland *et al.* (2006) have demonstrated a favorable effect on these physiological derangements, that leads to an improvement in clinical outcomes, including improved survival of critically-ill patients in the intensive care setting. Likewise, the observation by Cavalcante *et al.* (2012) that patients receiving glutamine supplementation are likely to sustain antioxidant capacity reflects the role of glutamine as a precursor of GSH (Cavalcante *et al*., 2012; Heyland *et al*., 2006).

Intravenously L-alanyl-glutamine (50 g) administered three hrs prior to surgery in patients subjected to critical limb ischemia decreased TBARS levels and increased GSH concentrations in the muscle 0.5 hrs after reperfusion following venous grafting. This pretreatment also reduced levels of LDH and lactate, indicating increased glucose oxidation in the limb submitted to ischemia/reperfusion, suggesting that glutamine reduced glycemia in the patients. It may therefore be concluded that pretreatment with L-alanyl-glutamine reduces muscle cell damage and enhances antioxidant capacity in patients with critical limb ischemia (Alves *et al*., 2010).

Glutamate replacement may be achieved by means of exogenous glutamine supply, thereby maintaining the intracellular glutamate pool and avoiding glutamate depletion (Alves *et al*., 2010).

12.5 Intestinal barrier

Intestinal barrier function depends on a balance between epithelial cell (enterocyte) proliferation and apoptosis and between protein synthesis and proteolysis, but also on the maintenance of the junctions between enterocytes, the so-called 'tight junction' (Coëffier *et al*., 2009).

The intestinal mucosa plays a key role in intestinal barrier function, preventing systematic dissemination of bacteria and intraluminal endotoxins. However, under conditions of severe damage, anatomical and functional integrity may be lost, allowing bacterial translocation and, consequently, sepsis and multiple organ failure (Francis and Michel, 2000).

Bacterial translocation implies the transfer of viable endogenous bacteria from the gastrointestinal tract to extra-intestinal sites, such as the mesenteric lymph node complex, the liver, the spleen or the blood. Among the factors implicated in this condition are long-term nutritional deficits during severe disease; changes in gastrointestinal microflora, leading to bacterial overgrowth; physical disruption of the intestinal mucosal barrier through direct damage to enterocytes, thereby favoring penetration; reduced intestinal blood flow; and local or systemic response, causing deficiencies in the immune defense and increased permeability or damage to the intestinal mucosal barrier (Grimble, 2001; Wiest and Rath, 2003).

Thus, the inflammatory response to injury and infection, although an essential part of immune function, carries the risk of severe tissue depletion and immunosuppression (Grimble, 2001).

Bacterial translocation can occur in both healthy and diseased individuals regardless of disease severity, but tends to increase in severe clinical conditions, such as burns, trauma, surgery and heart failure. Intestinal barrier permeability can be reduced to prevent bacterial translocation by different treatment approaches, including enteral nutrition and immunonutrition (Coëffier *et al.*, 2009; Grimble, 2001; Wiest and Rath, 2003).

12.6 Immunonutrition

Immunonutrition can modulate the immune system, or the consequences of immune system activation, by supplying specific foods and nutrients in amounts exceeding those found in conventional diets (Grimble, 2001, 2009). Glutamine is an immunomodulate amino acid capable of maintaining intestinal integrity and stimulating protein synthesis in human intestinal mucosa (Coëffier *et al.*, 2009; Francis and Michel, 2000).

Nutritional glutamine depletion has been associated with reduced glutamine levels in plasma and mucosa and with increased intestinal permeability. In a study on glutamine plasma concentrations and nutritional parameters in 26 patients receiving parenteral nutrition, the inflammatory activity occurred independent of the presence or absence of nutritional depletion (Hulsewé *et al.*, 2004).

Several studies on enteral, parenteral or oral nutritional therapy recommended doses of glutamine before or just after surgery and burns and have showed the glutamine protective effect preventing or reducing loss of intestinal permeability, maintaining the physiological intestinal barrier and reducing the frequency of infection.

12.7 Glycolytic metabolism

Hyperglycemia is multifactorial and is associated with a poor prognosis in clinical or surgical ICU patients. In this setting, hyperglycemia develops mainly due to gluconeogenesis, insulin resistance, up-regulation of insulin counter regulatory hormones, cytokine activity, clinical interventions and glucose intolerance (Van den Berghe *et al*., 2006).

Common in sepsis (even in non-diabetic patients), hyperglycemia causes or exacerbates oxidative stress, endothelial injury, neutrophil rolling and inflammation and has pro-inflammatory and immunosuppressive action (Kumar and Leaper, 2005).

As an alternative to therapy with exogenous insulin supply, studies have shown that nutritional glutamine supplementation can attenuate insulin resistance and subsequent hyperglycemia following severe disease. Clinical study on glutamine supplementation to severe and polytraumatized patients revealed a reduction in infectious complications and improved metabolic tolerance in a group of patients requiring insulin therapy (Bakalar *et al*., 2006; Dechelotte *et al*., 2006).

In a recent study it was found that potential benefits of parenteral alanyl-glutamine supplementation (0.3-0.6 g/kg/d) in glucose homeostasis are associated with a lower incidence of hyperglycemia among critically ill polytrauma patients, and leads to a lower mean daily dose of insulin (Grintescu *et al*., 2014).

Type 2 diabetes is a major risk factor for cardiovascular disease development. A study demonstrated that the long-term consumption of 10 g of glutamine three times a day for six weeks with main meals markedly reduced glycemia, high blood pressure, waist circumference and improved body composition in type 2 diabetes. Glutamine may be a factor contributing to reduction of complications in type 2 diabetes (Mansour *et al*., 2015).

Based on these current studies (Grintescu *et al*., 2014; Mansour *et al*., 2015) and past studies (Bakalar *et al*., 2006; Dechelotte *et al*., 2006), it seems reasonable to affirm that glutamine has beneficial effects on insulin-dependent glucose metabolism.

12.8 Glutamine and enteral nutrition

Nutritional therapy can be used as a tool to modulate health outcomes, improving the clinical and biochemical indicators affected by the disease. One such tool, enteral nutrition, is indicated for patients with conditions which preclude normal oral feeding and, consequently, nutrient absorption. It provides necessary calories and substrates and restores metabolic and immune response, improving host defense and accelerating recovery.

Over the past decades, enteral nutrition has become a vital component in intensive care. It is in most cases the recommended strategy of feeding severely ill patients presenting increased nutritional requirements and, uncommonly, catabolism with risk of organ failure. In addition, ICU patients often experience depletion of specific nutrients, such as arginine, minerals and glutamine.

In enteral diets, glutamine supplementation is safe and well tolerated and can help reduce infectious complications, oxidative stress, intestinal permeability, infections, mortality and morbidity rates and hospitalization cost and time (Houdijk *et al*., 1998), potentially benefiting patients with severe SIRS (Conejero *et al*., 2002), cancer (Lim *et al*., 2009; Senkal *et al*., 2004), sepsis and infection (Cavalcante *et al*., 2012).

Glutamine may act as a pharmaconutrient at nutraceutical doses (10-50 g/d). Under such conditions, it has specific and important metabolic functions, especially in injury related to infection or wounds or in catabolism and sepsis. The rapid depletion of glutamine in the plasma, which is commonly observed in critically ill patients, appears to justify the need of replacement by way of supplementation (Abilés *et al*., 2008; Fuentes-Orozco *et al*., 2004; Novak *et al*., 2002; Senkal *et al*., 2004).

Enteral glutamine administration allows its use initially in splanchnic tissues, whereas parenteral supplementation provides glutamine for the entire organism (Biolo *et al*., 2005).

The effect of glutamine-enriched enteral nutrition on intestinal permeability, infectious morbidity and mortality investigated in severe patients developing SIRS following an acute event showed that glutamine-enriched enteral nutrition reduced nosocomial infections in this population (Conejero *et al*., 2002).

Careful study reviewed several studies and found that enteral glutamine supplementation at nutraceutical doses was safe and well tolerated clinically, with no adverse effects regardless of the disease (García-de-Lorenzo *et al*., 2003).

Galera *et al*. (2010) evaluated the safety profile of oral administration of l-glutamine at 30 g/d for 14 days in a sample of healthy middle-aged and elderly subjects and observed no clinically important effects, apart from the need for permanent control of renal function in this population. Likewise, this finding showed that glutamine oral supplementation at nutraceutical doses seems to be safe (Fuentes-Orozco *et al*., 2004; Galera *et al*., 2010; Garcia *et al*., 2003).

Macrophages and lymphocytes are the largest cell components of the immune system. The latter are a group of heterogeneous cells involved in immune cell mediation and immunoregulation. Evaluating the effect of nutraceutical dose of L-glutamine (30 g/d) in moderately severe adult and elderly ICU patients with sepsis, Cavalcante *et al*. (2012) observed an increase in the total number and percentage of lymphocytes induced by L-glutamine enteral nutrition supplementation.

Additionally they observed a decrease in lipid peroxidation, with either supplementation of L-glutamine or calcium caseinate, but had no effect on glycolytic parameters or inflammatory response (Cavalcante *et al*., 2012).

Glutamine-enriched diets at recommended nutraceutical doses (20-30 g/d) should be started as early as possible and maintained for at least 5 days (Garcia-de-Lorenzo *et al*., 2003). Since the turnover of glutamine is very high, a daily dose of 30 g may be administered with no risk or significant side effects (Cavalcante *et al*., 2012; Galera *et al*., 2010).

This findings suggested that glutamine, despite being supplied enterally, increased the percentage of lymphocytes, indicating the amino acid was not retained by the enterocytes. Yet, the finding of an increased percentage and total number of lymphocytes indicates significant improvement of the immune system, especially when considered in combination with a significant decrease in the percentage of leucocytes, as observed in both study groups.

On the other hand, in a study administering parenteral nutrition supplemented with l-alanyl-glutamine at 0.4 g/kg/d to severe patients with APACHE II scores from 22 to 26, no significant difference was observed with regard to the total number of lymphocytes, B-lymphocytes and T-lymphocytes or their subgroups (helper T-lymphocytes, cytotoxic T-lymphocytes) (Cetinbas *et al*., 2010).

Regarding the findings of these last two studies, it seems that some clinical patients do not benefit as much from glutamine supplementation as other patients. A possible explanation for the absence of an effect of treatment with glutamine supplementation could be a significant number of limitations in the trials, e.g. small samples, random allocations, length of supplementation period and nutraceutical dose used.

However, evidence supports glutamine as an ideal pharmacologic intervention in the ICU population and can be recommended as a standard of care for all patients (Weitzel and Wischmeyer, 2010).

Considering the observed benefits and the absence of adverse effects, enteral and parenteral diets enriched with l-glutamine or l-alanyl-glutamine are currently recommended for critical ill patients in ICU setting in order to modulate the immune system.

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13. The role of the neutropenic diet in preventing infection in cancer patients on chemotherapy: a critical review

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Abstract

Chemotherapy has had a major impact on the survival rates of patients with cancer. However neutropenia due to chemotherapy is the major risk factor for infection. We therefore reviewed the literature to determine whether neutropenic diets decrease the incidence of infection in patients undergoing chemotherapy. A systematic review of the literature including retrospective and randomized clinical trials published between 1966 and 2015 was performed. Data sources included Medline, Embase, Pubmed, and the Cochrane Central Register of Controlled Trials. The Cochrane review of randomized clinical trials comparing the use of neutropenic vs. control diet concluded that no conclusion could be drawn. A large retrospective study comparing a neutropenic and control diet in patients undergoing hematopetic stem cell transplant indicated a similar finding. Unanswered questions include: which foods should be included, food preparation techniques to improve patient compliance, which patient populations benefit most, and when should such a diet be initiated. Until these questions are addressed, the best advice for neutropenic patients is to follow food safety guidelines by government agencies.

Keywords: neutropenia, low bacterial diet, clinical trials, USDA guidelines

Key facts:

- Neutropenia due to chemotherapy is a major risk factor for infection.
- Bacterial and fungal infections can result from food and water sources.
- Food is important in patients' quality of life.
- Both randomized and retrospective studies comparing a neutropenic diet to a normal diet have revealed no difference in the incidence of infection.
- Since there is no of cial published guidelines on the use of the neutropenic diet, following the United States Department of Agriculture recommendations of avoiding uncooked meat, seafood, and unwashed fruits/vegetables may be prudent.

Summary points

- Neutropenia due to chemotherapy is the major risk factor for infection.
- Bacteria (especially Gram-negative organisms) and fungal infections (e.g. *Aspergillus*) can result from food and water sources.
- There is no standard definition for the neutropenic diet. Variations of the diet may include well-cooked foods only or a regular diet omitting fresh fruits and vegetables.
- Although the benefit of the neutropenic diet is unclear, such a diet is used in many institutions.
- Both four small randomized and several retrospective studies comparing the use of a neutropenic vs. control diet revealed no difference in the incidence of infections.
- There are no of cial published guidelines on the use of the neutropenic diet.
- Several randomized trials comparing a neutropenic diet to a standard diet are ongoing.
- Several studies have emphasized the importance of food in patients' quality of life.
- In neutropenic patients with cancer undergoing chemotherapy, basic principles such as avoiding uncooked meats, seafood, eggs and unwashed fruits/vegetables may be prudent.

Abbreviations

ALL	Acute lymphoblastic leukaemia
AML	Acute myelogenous leukaemia
ANC	Absolute neutrophil count
ERM	Early risk of mortality
FDA	Food and Drug Administration
GI	Gastrointestinal
GVHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplantation
LBD	Low bacterial diet
NCCN	National Comprehensive Cancer Network
ONS	Oncology Nursing Society
OR	Odds Ratio
USDA	United States Department of Agriculture

13.1 Introduction

Chemotherapy has had a major impact on the survival rates of patients with cancer, particularly those with hematologic malignancies (Sausville and Longo, 2012). However, neutropenia due to chemotherapy is the major risk factor for infection (Bodey *et al.*, 1966), particularly if prolonged and associated with intense chemotherapy for hematologic malignancies and with stem cell transplantation. Bacteraemia may occur in approximately 15 to 25% of patients, most often when the ANC is less than 500/μl (Fox and Freifeld, 2012) or when there is a rapid fall in neutrophil count.

Bacteraemia may result from food and water sources. Several studies (Casewell and Phillips, 1978; Pizzo *et al.*, 1982) have reported the isolation of Gram-negative organisms such as *Pseudomonas aeroginosa, Escherichia coli, Klebsiella,* and *Proteus* from a variety of foods, particularly salads, fresh vegetables, and cold meats. *Aspergillus*, a fungus often lethal to patients with prolonged neutropenia, was found and isolated from food, water, and ice (Thio *et al.*, 2000).

The movement of these bacteria from the GI tract to other body sites, called bacterial translocation (Berg, 1999), is thought to cause many of the infections in patients with neutropenia. Deitch *et al.* (1987) demonstrated that bacteraemia in the GI tract can travel through the intestinal mucosa to infect mesenteric lymph nodes and body organs. Bacterial overgrowth, immunosuppression, physical disruption of gut by chemotherapy or radiotherapy, slowed peristalsis due to narcotics or anti-diarrheal agents, trauma, and endotoxins are factors contributing to bacterial translocation (Carter, 1994). Theoretically, bacterial translocation can be decreased by reducing sources of pathogenic bacteria from food and decreasing the bacterial burden of the gut with oral non-absorbable antibiotics.

The above studies led to the use of neutropenic diet. The neutropenic diet is also called a sterile diet, low microbial diet, or a LBD. However, a standardized definition of the neutropenic diet has not been established (DeMille *et al.*, 2006; Smith and Besser, 2000). Variations of the neutropenic diet include an exclusively sterile diet (e.g. all foods that have been made sterile by canning, baking, autoclaving, or irradiation), a LBD (well-cooked foods only), or a modified house diet (i.e. a regular diet omitting fresh fruits and vegetables) (DeMille *et al.*, 2006; Moody *et al.*, 2002; Smith and Besser, 2000; Todd *et al.*, 1999).

Although the benefit of the neutropenic diet has never been scientifically proven, neutropenic diets are still used in many institutions. A descriptive telephone survey by Todd *et al.* (1999) looked at the use of LBDs for chemotherapy-induced neutropenia among 21 children's hospitals and found that 43% of these hospitals used the neutropenic diet for neutropenic non-bone marrow transplant patients and 86% of these hospitals used the neutropenic diet for bone marrow transplant patients. French *et al.* (2001) surveyed ten bone marrow transplant centres in Canada and North-western USA and reported that five of the seven responding hospitals used a neutropenic diet. However, the timing of the start of the diet and the food choices that were allowed varied with each institution. Poe *et al.* (1994), found that 66% of responding transplant units enforced some type of modified microbial diet. Smith and Besser (2000) surveyed 400 members of the Association of Community Cancer Center and reported that 78% of the responding hospitals restricted diets of patients with neutropenia. The most commonly prohibited food items in these institutions were fresh vegetables and fruits, fresh juices, and raw eggs. Criteria for dietary restrictions varied between hospitals. The most common reason for restricted diets were documentation of neutropenia defined as a white cell count <1000 mm (Fox and Freifeld, 2012) and documentation of risk factors for neutropenia, such as recent chemotherapy.

A self-administered electronic survey was sent to 1,639 pediatric oncologists who are members of the Children's Oncology Group to determine the percentage of those who use the neutropenic diet and to determine which factors influenced their decisions to use such a diet (Braun *et al.,* 2014). 557 physicians (34%) responded, 57% of who reported implementing the neutropenic diet. In a multivariate analysis, the only significant factors associated with implementing a neutropenic diet was being a stem-cell transplant center (OR 6.6, 95% CI 2.88-12.738, $P<0.001$) after controlling for years in practice, gender, center size, and academic vs. private practice. Among those who implemented a neutropenic diet, the ANC was the trigger for 72% of oncology patients and for 84% of transplant patients starting a preparative regimen. 82% of respondents stop the neutropenic diet when the oncology patients are no longer neutropenic. For those who initiated the diet based on ANC, 89% initiated it when the ANC was <500, 5% when the ANC is <1000, and 5% when ANC <1,500. Among those who use the neutropenic diet, the most restricted foods included fruits that cannot be peeled, raw vegetables, herbs, sprouts, unpasteurized dairy products, aged cheeses, well water, sushi, and raw honey.

13.2 Published studies on the neutropenic diet and incidence of infection in cancer patients

The current rationale for recommending a neutropenic diet is based on the prospective cohort of randomized studies performed in the 1960's and 1970's in which leukemia patients were placed in a total protective environment in the hospital setting (Moody *et al.*, 2002) (e.g. isolation tents, use of oral non-absorbable antibiotics, laminar air flow, and sterile diet). In this controlled environment, patients were found to tolerate higher doses of chemotherapy with less toxicity, including secondary infections. Although these early studies suggested that protected environments might offer some safeguard from infections, the independent effect of the neutropenic diet on infection rates was unclear.

The published studies are summarized in the subsequent paragraphs. Data sources include Medline, Embase, Pubmed, and the Cochrane Central Register of Controlled Trials. Four randomized controlled trials and two retrospective studies were found and all are discussed below. Two of the studies involve the hematopoietic stem cell population.

A study by Österblad *et al.* (1999) attempted to answer the question of whether or not food ingested by neutropenic patients is a source of pathogenic bacteria that ultimately causes bacteremia or other serious infections. They found that certain bacteria (e.g. *Enterobacteriaceae*) cultured from the surfaces of vegetables had a significantly different antibiotic susceptibility profile than the *Enterobacter* species cultured from human stool samples. The conclusion was that the antibiotic resistant bacteria from vegetables probably do not play a significant role in colonizing the human gut.

In a study by Moody *et al.* (2006), 19 pediatric patients receiving myelosuppressive chemotherapy were randomized to a neutropenic diet or to the FDA approved food and safety guidelines diet. Patients included those being treated with chemotherapy for malignant brain tumors, ALL, or sarcoma. Patients randomized to the neutropenic diet were given dietary restrictions including no raw fruits (except for those that could be peeled by hand), no raw vegetables, aged cheeses, cold meat cuts, fast food, or takeout food. For the most part, all patients on the food safety guidelines diet adhered to the diet while adherence was 94% for the neutropenic diet. The mean number of days of neutropenia (ANC <500/l) was 5.9 days for the neutropenic diet arm (n=10) and 9.2 days for the food safety arm (n=9) which was not significant (P=0.29). Four patients in each arm developed neutropenic fever. There was one episode of bacteremia (*P. aeruginosa*) in the neutropenic diet arm but none in the food safety cohort.

DeMille *et al.* (2006), sought to determine whether the use of the neutropenic diet (no fresh fruits and vegetables) in an outpatient setting influenced the number of febrile admissions and positive blood cultures. 23 patients age 33 to 67 years completed a 12-week program in which they were instructed on the neutropenic diet prior to chemotherapy. Study personnel used phone calls to assess adherence at 6 and 12 weeks and reviewed hospital charts at the end of the study. 16 patients were deemed compliant while seven were non-compliant. Four (25%) from the compliant group

had a febrile admission, three of whom had gram-negative bacteremia, whereas one (14%) of the non-compliant group had a gram-negative febrile admission.

In a study by Van Tiel *et al.* (2007), 20 adult patients with both ALL and acute myeloid leukemia receiving remission-induction chemotherapy were randomized into two groups: one group to receive antimicrobial prophylaxis (Cipro) and a LBD; and the other group to receive the same antibacterial prophylaxis and a normal hospital diet. During chemotherapy cycles, fecal samples were collected daily to detect gram-negative bacilli or *Candida* species. There were no differences in the two groups with respect to mean age, mean weight, total number of chemotherapy cycles, and total number of days within chemotherapy cycles. There were no statistically significant differences between the treatment groups regarding the incidence of stool colonization by yeasts and gram-negative bacilli, confirmed and unconfirmed infections, number of days of antibiotic use and calculated total medical costs.

In the largest study to date, Gardener *et al.* (2008) studied 153 newly diagnosed AML patients over a period of three years (2004-2007) who were admitted to a high-efficiency particulate air-filtered room to receive induction therapy. With use of their ERM score for stratification, patients were randomly assigned to a diet with (uncooked; n=75) or without (cooked; n=78) fresh fruits and vegetables. All fresh fruits and vegetables were washed thoroughly with cold water, always kept refrigerated and delivered immediately to the patient's room. All patients were advised not to eat fast food, soft-serve ice cream, deli meats, raw fish, or fresh salad-dressing from the refrigerated section, and unpasteurized foods. Patients were also advised not to eat at buffets and not to leave food out of the refrigerator for several hours. Prophylaxis with both antibacterial and antifungal agents was used for all patients. No differences were found between the groups for age, ERM score, chemotherapy received, or days at risk. The study outcomes showed no significant difference for time to major infections (P=0.44), or survival (P=0.36). The proportion of patients who developed a major infection was 29% for those in the groups without fresh fruits and vegetables and 35% for those allowed to have fresh fruits and vegetables (P=0.6). Fevers of unknown origin developed in 51% of the cooked and 36% of the raw group (P=0.07).

There are major limitations to most of these studies. These include the following: (1) small sample size; (2) the absence of documentation and/or measurement of other variables that determine the incidence of neutropenic infections including the degree and duration of neutropenia, exposure to viruses, use of granulocyte-colony stimulating factor, hematologic vs. solid tumors, and extent of mucositis; and (3) potential study bias due to awareness of the neutropenic diet by patients assigned to the regular diet arm, resulting in their reluctance to eat fresh fruits or vegetables.

In a Cochrane review (Van Dalen *et al.*, 2012) of randomized controlled trials comparing the use of a neutropenic vs. a control diet, Van Dalen *et al.* (2012) identified three randomized studies in 192 children and adults with different types of cancer. The conclusions of this review were that: (1) there was no evidence from the individual studies showing that the use of the LBD prevents infections; (2) data on survival, time from onset of neutropenia to onset of fever, the duration of prophylactic antibiotics and antimycotics, diet acceptability (i.e. following the diet easily and

following the diet throughout all chemotherapy cycles), and quality of life were all evaluated in only one study; (3) none of the studies provided a detailed description of treatment regimens; (4) none of the studies evaluated infection-related mortality; and (5) no recommendations for clinical practice based on the current evidence could be given.

A recent retrospective study by Trifilio *et al.* (2012), investigated the role of the neutropenic diet in preventing infections in patients treated with HSCT. The investigators studied 726 consecutively treated stem cell transplant patients; 363 received a neutropenic diet and 360 received a general hospital diet. The neutropenic diet excluded all fresh fruits and vegetables as well as black pepper, raw and undercooked meats and cheeses, cold smoked fish, raw or unpasteurized dairy products, and brewer's yeast. The general diet mandated safe food handling practices and permitted black pepper and well-washed fresh fruits and vegetables but excluded raw tomatoes, seeds, and grains. The other restrictions remained. The neutropenic diet was initiated around the time of neutropenia but once neutropenia resolved, the general diet was initiated (except those with GI GVHD). An autologous HSCT was performed in 543 patients and allogeneic HSCT in 183 patients. The most common diagnoses were multiple myeloma, non-Hodgkin's lymphoma, AML, Hodgkin's disease, and ALL. The results of the study, which was conducted over a four-year period, revealed 135 microbiologically confirmed infections in the neutropenic diet group and 106 confirmed infections in the general diet group. There were significantly fewer microbiologically confirmed infections therefore, in the general hospital diet groups ($P<0.0272$), which was especially evident after the resolution of neutropenia ($P<0.008$). Diarrhea was more common in the general diet group ($P<0.095$) as were urinary tract infections ($P<0.003$). Acute grade II-IV GI GVHD was more frequent in the neutropenic diet group post-transplantation. Overall survival did not differ between the two treatment groups. However, the above study had the following limitations: (1) the majority of the transplant patients received autologous transplants and these patients typically have a shorter duration of neutropenia (i.e. 10 to 14 days); (2) the lack of accounting for the degree of gut toxicity of the conditioning regimens; (3) the single center experience; (4) the low incidence of some infections (e.g. molds); and (5) the non-randomized nature of the study. Despite the above limitations, the sample size was large and one of only two published studies conducted in HSCT recipients to evaluate the effectiveness of neutropenic diet in preventing hospital infections.

The other study, by Lassiter and Schneider (2015), was a randomized controlled pilot study comparing a neutropenic diet (only cooked food and thick skinned fruits) to a regular diet in 46 people who underwent allogeneic HSCT for hematologic malignancies. All of the patients received care in a high-efficacy particulate air filter room. All of the patients had a tunneled central venous catheter placed prior to beginning the conditioning regimen. All patients received prophylactic ciprofloxacin, metronidazole, acydovir, fluconazole, and voriconazole (for umbilical cord transplants). With the first neutropenic fever, all patients were started on IV vancomycin and ceftazidine. No differences were found between the groups in the percentage of positive blood cultures.

13.3 Are there published guidelines on the use of the neutropenic diet?

Because there is an absence of evidenced-based studies supporting the use of the neutropenic diet, there are no official published guidelines on its use. The NCCN (2015) guidelines on prevention and treatment of infectious complications do not mention the use of the neutropenic diet. The ONS (2005) cancer chemotherapy guidelines and recommendations for practice state that, 'no recent studies have linked dietary restrictions with a lower risk of infection for neutropenic patients with cancer; however, basic principles, such as avoiding uncooked meats, seafood, eggs, and unwashed fruits and vegetables may be prudent.

The Infectious Disease Society of America 2010 update of clinical practice guidelines for the use of antimicrobial agents (Freifeld *et al.*, 2011) in neutropenic patients with cancer state that 'well-cleaned, uncooked raw fruits and vegetables are acceptable, as are cooked foods brought from home or restaurants provide that the freshness of ingredients and the means of preparation can be confirmed.

The USDA (2011) recommendations for food safety for people with cancer include the following: (1) consumption only of pasteurized juices and dairy products; (2) washing hands in warm soapy water before handling, preparing, and eating food; (3) consuming food that has not passed the expiration date; and (4) storing raw meat, fish, and chicken carefully in wrapped containers to avoid spillage of juice onto other foods. Notably, there is no recommendation for the restriction of fresh fruits and vegetables. More recently, the FDA has recommended avoiding raw sprouts (e.g. alfalfa, bean, clover) (Fox and Freifeld, 2012). The warm moist conditions in which sprouts are produced are an ideal environment for the growth of bacteria, such as *Salmonella, Listeria*, and *E. coli*.

13.4 Conclusions

The authors of the Cochrane review of the neutropenic diet (Van Dalen *et al.*, 2012) indicated that 'no evidence of effect' is not the same as 'evidence of no effect' and that more high quality research is needed. Research questions to consider might include the following:

- Which food choices and food preparation techniques would best improve patient compliance?
- Is there a specific oncology population who benefits most from the use of the neutropenic diet?
- Is there a role for the neutropenic diet in neutropenic chemotherapy patients independent of other interventions, i.e. antibiotics, growth factors, and the use of private rooms to prevent infections?
- Should the neutropenic diet be initiated at the start of chemotherapy or only when neutropenia develops?
- Are there losses of nutrients in the preparation of the neutropenic diet? Several studies have reported a significant reduction in B vitamins, potassium, and vitamin C during the cooking process including soaking, boiling, cooking in a microwave, and pressure cooking (Galati *et al.*, 2013; Garófolo, 2013).

- Which of the strategies – i.e. low or no bacterial diet to try to prevent neutropenic fever vs. a no enteric pathogen diet to try to prevent infections such as salmonellosis, and listeriosis result in a better outcome with respect to quality of life, incidence of infection, or overall mortality?
- Should fruits and vegetables be washed with sterile water in light of recommendations that fruits and vegetables to be eaten raw should be washed thoroughly?

In developed countries water treatment results in tap water which may contain many microbes. Water distribution systems may contain pathogens such as *Legionella* sp., *P. aeruginosa, Stenotrophomonas maltophilia* and other Gram-negative organisms, and *Cryptosporidium* spp. (Decker and Palmore, 2014; Lund 2014).

Several randomized trials comparing a neutropenic diet to a standard diet in patients with cancer undergoing chemotherapy are presently ongoing, according to the www.clinicaltrials.gov database. One study was initiated by Moody *et al.* (2011) (Montifiore Hospital, New York) to compare the FDA approved food safety diet to the neutropenic diet with respect to the rate of infection during neutropenia in patients aged 1-30 years receiving chemotherapy for ALL, AML, sarcoma, non-Hodgkin's lymphoma, and neuroblastoma. The neutropenic diet in this study emphasizes the avoidance of the following: raw vegetables and fruits except oranges and bananas; takeout food and fast foods; aged cheese (blue, Roquefort, and Brie); deli meats; raw nuts or nuts roasted in shell; well water; and yogurt. An estimated 400 patients were to be enrolled. The study is still ongoing.

A randomized pilot study recently completed by Chao *et al.* (2013) (Duke University) compares infection rates in myeloablative allogeneic stem cell transplant patients age 20-70 receiving a non-neutropenic diet or a neutropenic diet. The assigned diet will continue until the subject is no longer neutropenic (defined as an ANC of greater than 500 per μl) and a total white cell count of 1000 per μl for three consecutive days. The details of the neutropenic diet were not available.

Although the available evidence does not support use of the neutropenic diet, some argue that it is prudent to be cautious and continue to provide immune compromised patients on a neutropenic diet. A recent paper by Lund (2014) reported a number of recent outbreaks of food associated microbiological illness in immunocompromised patients. These include: *Clostridium perfringens* in cooked meat products that are then maintained with inadequate cooling; *Salmonella,* Shiga toxin producing *E. coli* (STEC), and *Norovirus* in fresh (salad) vegetables; *Salmonella* infections associated with tomatoes, peppers and melons; the parasite *Cyclospora* associated with raspberries; *Norovirus* and hepatitis A virus associated with frozen raspberries, strawberries, and blackberries; *Listeria monocytogenes* associated with cantaloupes; and *Aspergillus fumigatus* and *Aspergillus flavus* associated with spices, particularly black pepper.

However, several studies have emphasized the importance of food in patients' quality of life (Moody *et al.*, 2002; Moody *et al.*, 2006). Patients receiving chemotherapy experience many stressors including body image changes, and an uncertain future (DeMille *et al.*, 2006). Thus

patients identify appetite and weight as variables within their control, and food is seen as a nurturing and comforting area of life (DeMille *et al.*, 2006). Since there remains considerable lack of clarity as to what, if any, restrictions on diet neutropenic patients should be advised to follow, it seems prudent to follow the guidelines regarding safe food handling (USDA, 2011), as recommended by the USDA, in both improving the quality of life and aid in the prevention of life-threatening infections in oncology patients.

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14. Taurine affects hematologic properties and diseases

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Abstract

Taurine is a semi-essential amino acid found abundantly in all mammalian cells. Taurine plays diverse functions from prenatal to adult life. Compared to plasma, blood cells, particularly neutrophil, lymphocyte, and platelet, are rich in taurine, while red blood cells contain lesser taurine. Taurine involves blood cell synthesis, growth, differentiation, maturation, stabilization, optimal function, and apoptosis. In neutrophils and monocytes, taurine possesses anti-oxidative activity by reacting with hypochlorous acid to form taurine chloramine. Although cell oxidative stress is one of the important processes for pathogen destruction by leucocytes, too much oxidative stress limits the phogocytic activity and accelerates leucocytic apoptosis. Taurine chloramine is a lesser oxidant which optimizes the phagocytosis and delays cell apoptosis. In addition, taurine and its derivatives improve cell-mediated and humoral immunity, as supported by both *in vitro* and *in vivo* experiments. In platelets, taurine stabilizes platelet activity and prevents platelet aggregation. Thus, taurine deficiency in either perinatal or adult life alters hematologic properties and impairs immune function, while taurine supplementation or a diet high in taurine improves human health and physical performance. These lines of evidence allow taurine to be an important component in energy drinks and healthy nutritional products. However, clinical applications of taurine are still limited and need to be studied more in humans.

Keywords: blood, hematology, immune, leucocyte, oxidative stress, platelet

Key facts:

- Taurine plays many important roles throughout life.
- Taurine deficiency impairs hematologic properties.
- Taurine is commonly found in many foods.
- Taurine improves innate and adaptive immunity.
- Taurine prevents thrombosis.

Summary points:

- Blood cells are rich in taurine.
- Taurine possesses anti-oxidation, anti-apoptosis, and anti-thrombosis properties.
- Taurine improves innate and adaptive immunity.
- Taurine supplementation is clinically relevant.

Abbreviations

CD3/4/8/25	Cluster of differentiation 3/4/8/25
Fas(CD95)	Fas receptor
FasL	Fas ligand
H_2O_2	Hydrogen peroxide
IL	Interleukin
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
TNF-α	Tumor necrosis factor α

14.1 Introduction

Inflammation and immune dysfunction underlie many diseases including diabetes mellitus, hypertension, kidney disease, neurologic disorder, and obesity. Furthermore, several disorders and treatments affect hematologic properties and blood chemistry, e.g. cancer and chemotherapy. All blood cells are derived from hematopoietic stem cells in bone, liver, and lymph organs and these organs produce and destroy blood cells to match the need of the body throughout life, beginning at fetal stages. Appropriate hematopoiesis, differentiation, maturation, function, and apoptosis are a complex mechanism involving both internal and external factors. Taurine is an important component of blood cells and plays a crucial role in hematologic function. Insufficient taurine availability during critical stages can lead to hematologic disorders and related diseases.

This chapter focuses the role of taurine on hematologic properties and related disorders. Both perinatal and adult effects of taurine depletion and supplementation are reviewed.

14.2 Physiology of taurine

Taurine (2-aminoethanesulfonic acid) is a β-amino sulfur amino acid found in many tissues including blood cells. In general, taurine content in the body is highest during early postnatal life and declines with advancing age (Roysommuti and Wyss, 2014). Taurine supplementation increases taurine content in many organs. During pregnancy, taurine accumulates in maternal tissues is released to the fetus via the placenta and to the newborn via the maternal milk. Inadequate maternal taurine availability results in prenatal and early postnatal taurine deficiency symptoms. In addition, perinatal taurine exposure affects adult function and disease.

Taurine plays diverse physiological functions beginning at conception and continuing throughout life (Roysommuti and Wyss, 2013). Taurine is not only well-known as an organic osmolyte in all mammal cells, but taurine also possesses anti-hyperglycemic, anti-hyperlipidemic, anti-hypertensive, anti-inflammatory, and anti-oxidative effects. In addition, taurine inhibits the renin-angiotensin system and sympathetic nervous system and modulates the immune system

(Figure 14.1). Perinatal taurine supplementation promotes prenatal and postnatal growth and development and protects against adult diseases, including hematologic disorders. However, taurine supplementation in late pregnant rats stimulates postnatal growth and induces obesity and insulin resistance in adult offspring (Hultman *et al.*, 2007). Further, our experiments indicate that both perinatal taurine supplementation and depletion alter renal function and arterial pressure control in adult rats (Roysommuti *et al.*, 2009a,b, 2010, 2015). Thus, it is likely that an appropriate taurine intake, particularly during perinatal period is beneficial to human life.

Several lines of evidence indicate that taurine or diets high in taurine can prevent or reduce hypertension in animal and human models (Roysommuti and Wyss, 2014). For examples, taurine supplementation attenuates hypertension in adult, spontaneously hypertensive rats. Sugar-induced hypertension also appears to be prevented by taurine treatment. In addition, epidemiological studies indicate that people with high taurine diets have a low incidence of hypertension and other cardiovascular diseases (Yamori *et al.*, 2010). While the effects of adult taurine exposure are modest, altered exposure during the perinatal period can have life-long effects on cardiovascular control (Roysommuti and Wyss, 2014).

14.3 Hematology and immune system

In human prenatal life, blood cell formation or hematopoiesis occurs mainly in the yolk sac during embryonic development (the first trimester of pregnancy), in spleen and liver during the second trimester, and bone marrow and lymph nodes from the third trimester throughout birth

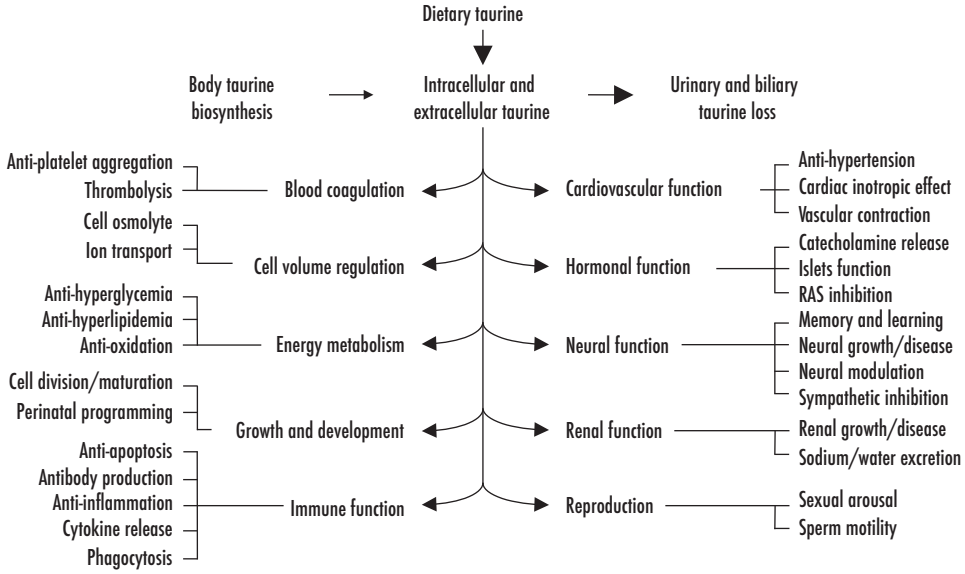

Figure 14.1. Taurine balance and function in humans (RAS, renin-angiotensin system).

and the rest of life. In adults, the bone hematopoietic stem cells or hemocytoblasts play a major role of hematopoiesis, while the lymph nodes are most important for blood cell differentiation and maturation, particularly the thymus gland for T lymphocytes and the gut lymph nodes for B lymphocytes. In children, hematopoiesis occurs mainly in the marrow of the long bones, e.g. the femur and tibia, while in adults, it occurs mainly in the pelvis, cranium, vertebrae, and sternum. There are four main types of blood cells; red blood cells (reticulocytes and erythrocytes produced solely by bone marrow stem cells), white blood cells or leucocytes (granulocytes including neutrophil, basophil, and eosinophil; agranulocytes including T and B lymphocytes, monocytes, macrophage, and dendritic cells), natural killer cells, and platelets (produced solely by bone marrow stem cell). These blood cells circulate in the blood and/or lymphatic system and some deposit in the tissue, e.g. macrophage and dendritic cells. Furthermore, they have many physiological functions from gas transport to blood coagulation (Table 14.1).

Table 14.1. Taurine content and function in different blood cells.

Cell type	Primary cell function	Taurine action in cell type
Neutrophil	Bacterial disinfection	Increase cell production
		Increase cell stabilization
		Increase phagocytosis
		Slow apoptosis after immune response
Eosinophil	Parasite disinfection	Increase cell stability
Basophil	Allergic response and inflammation	Anti-inflammation
T lymphocyte	Cell-mediated immunity	Increase cytokine release
		Increase cell proliferation
B lymphocyte	Humoral immunity	Increase antibody production
		Increase cell proliferation
Monocyte or Macrophage	Phagocytosis	Modulate cell proliferation
		Increase cell stabilization
		Increase phagocytosis
		Slow apoptosis after immune response
Natural killer cell	Phagocytosis	Increase cell proliferation
		Increase cell stabilization
		Increase phagocytosis
		Slow apoptosis after immune response
Platelet	Blood coagulation	Increase platelet production and stability
		Anti-platelet aggregation
Red blood cell	O_2 and CO_2 transport	Anti-oxidation
	Acid-base control	Maintaining apoptosis

Leucocytes and killer cells form a complex mechanism against invading pathogens (bacteria, viruses, fungi, and parasites), cancer cells, and other 'non-self' cells in our bodies. Innate immunity involves the non-specific role of physical factors (e.g. skin and gut), granulocytes, monocytes, macrophages, dendritic cells, natural killer cells, and complements, while adaptive immunity involves cell-mediated immune response by T lymphocytes and humoral immune response by B lymphocytes.

14.4 Taurine is crucial for hematologic and immune functions

Compared to plasma taurine levels (40-100 μM), blood cells, particularly platelets, eosinophils, and neutrophils, are rich in taurine (5-20 mM), while red blood cells contain much less taurine (<0.1 mM) (De *et al.*, 2001; Learn *et al.*, 1990). Taurine is reported to play an important role in immune function, and it has anti-platelet aggregation, anti-inflammatory, and anti-oxidant properties. In addition, taurine accelerates thrombolysis, but increases red blood cell stability (Gossai and Lau-Cam, 2009) *in vitro* at room temperature, taurine significantly enhances the stability of the platelet and red blood cell counts, mean corpuscular hemoglobin, and red blood cell distribution width (Sirdah *et al.*, 2013).

Cats cannot internally synthesize adequate taurine and need daily taurine from diets. Cats fed taurine-deficient diets for at least a year display leucopenia, an adverse shift in the percentage of polymorphonuclear and mononuclear leucocytes, an increase in the absolute count of mononuclear leucocytes, and a change in the sedimentation characteristics of white blood cells (Schuller-Levis *et al.*, 1990). Polymorphonuclear cells isolated from cats fed taurine-free diets decrease the respiratory burst and phagocytosis of *Staphylococcus epidermis* compared to cats fed the same diet containing taurine. However, serum γ-globulin in cats fed taurine-free (compared to taurine-supplemented) diets is significantly increased, indicating that B lymphocytes may be affected by chronic taurine deficiency. Histological examination of lymph nodes and spleen revealed regression of follicular centers with depletion of reticular cells, mature and immature lymphocytes (B cell areas), and mild extravascular hemolysis.

In 1992, Schuller-Levis and Sturman report that cats fed a taurine-free diet for 12 months display a decrease in the percentage of polymorphonuclear cells in the lungs and blood compared to those fed a taurine-supplemented diet. This result is consistent with a lower taurine content in the lung of taurine-deficient cats. Alveolar leucocytes from taurine-deficient cats produce more superoxide anion in response to phorbol myristate acetate than taurine-supplemented cats. Further, alveolar leucocytes of taurine-deficient cats produce more H_2O_2 than those of taurine-supplemented ones. These findings indicate that taurine is necessary for sufficient hematopoiesis and prevents leucocyte oxidative stress.

In taurine transporter knockout compared to wild-type mice, red blood cell and platelet counts are significantly lower and the packed cell volume is significantly smaller in taut-/- mice than in taut+/+ mice, while other erythrocyte parameters are similar. In contrast, white blood cell

count is significantly higher in taut-/- mice than in taut+/+ mice (Lang *et al.*, 2003). Plasma concentration and erythrocyte content of taurine is significantly lower in taut-/- than in taut+/+ mice, but the intra-erythrocyte taurine concentration does not exceed the plasma concentration. In addition, the taurine deficient mice increase resistance to hyperosmotic shock and oxidative stress, suggesting a resistance to apoptotic induction.

Intrauterine growth restriction is a well-known cause of adverse neonatal outcomes related to taurine deficiency. In preterm infants, numbers of white blood cells and neutrophils are diminished in small compared to appropriate for gestational age newborns at birth and on day three (Troger *et al.*, 2013). At birth, platelet counts are also lower in small vs. appropriate for gestational age infants, while the number of nucleated red blood cells tends to be elevated in small vs. appropriate for gestational age infants. After stimulation of whole blood cell cultures with lipopolysaccharides, the concentrations of IL-6 and IL-10 are significantly lower in small preterm infants compared to appropriate for gestational age infants. These findings indicate that innate immune function is suppressed in small compared to appropriate preterm newborns, and this may relate to a low taurine level in the preterm infants.

We have recently shown that both perinatal taurine depletion and supplementation affect hematologic properties in adult male rats (Vijitjaroen *et al.*, 2015). Female Sprague-Dawley rats were fed normal rat chow with 3% β-alanine (taurine depletion), 3% taurine (taurine supplementation) or tap water alone (control) from conception to weaning. Male offspring were fed with the normal rat chow and tap water *ad libitum* throughout the experiment. At 13-16 weeks of age, rats displayed similar body and kidney weights among the three groups. Compared to control and taurine depletion, taurine-supplemented rats displayed higher red blood cell count, hemoglobin concentration, and hematocrit. Mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red blood cell distribution width were not significantly different among groups. Perinatal taurine depletion and supplementation significantly decreased platelet count, while only the perinatal taurine supplementation (compared to control and taurine depletion) significantly increased mean platelet volume. Although total white blood cells, lymphocytes, monocytes, eosinophils, and basophils were not significantly different among experimental groups, neutrophils were significantly lower in taurine-supplemented compared to control but not taurine-depleted groups. The effect of perinatal taurine depletion in these studies is small compared to studies in taurine transporter knockout mice and taurine-deficient cats. This discrepancy may be due to the fact that the latter two models induce lifetime taurine depletion, while our model induces taurine depletion only during perinatal life. Further, many researchers also report that adult taurine supplementation can prevent several disorders in the adult offspring that are prenatally and/or early postnatally depleted of taurine. For example, intrauterine growth restriction induces low birth weight of newborns, and these offspring are prone to develop diabetes mellitus, obesity, and hypertension in adults. These effects can be prevented by taurine supplementation after weaning (Roysommuti and Wyss, 2014).

In normal adult Wistar rats, 60 days of oral taurine supplementation decreases platelet count, mean corpuscular hemoglobin, and % lymphocytes, but increases % neutrophils (Anand *et al.*, 2010). However, neutrophil phagocytic activity is significantly lowered by taurine supplementation. The similarity of the decrease in platelet count to our findings suggests that the hematological effect of perinatal taurine supplementation might not be due to high taurine levels in the adult, but rather to a programming effect of taurine in early life.

Taurine is a common component of many supplement foods and energy drinks. Other than taurine, most energy drinks are composed of caffeine, sugar, vitamin B_6 and water. Although the effect of taurine on the exercise performance is still unclear, taurine increases IL-6 and leucocyte number during exercise through early post-exercise in taurine-treated (compared to non-taurine control) subjects (Phillips *et al.*, 2014). However, the interpretation of the effects of taurine is somewhat confounded, due to interactions among caffeine, sugar, and vitamin B_6.

14.5 Taurine chloramine generation in leucocytes

Following infection, phagocytic leucocytes, particularly macrophages and neutrophils, act to destroy the invading pathogen-derived products (lipoproteins, nucleic acids, and peptides). Inside the phagocytes, several pathways help to destroy the pathogens and increase cell oxidative stress. In the neutrophils and monocytes, NADPH oxidase generates superoxide, which either dismutates spontaneously to H_2O_2 or is reduced more efficiently by the enzyme family of

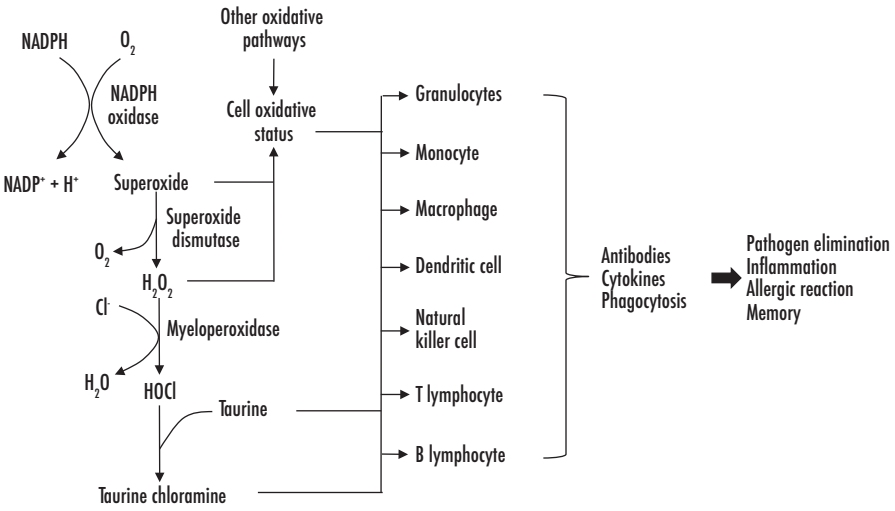

Figure 14.2. Taurine chloramine production in leucocytes and its immune function. Taurine chloramine appears to be the most powerful active derivative of taurine acting on the immune system, but the influence of taurine chloramine on the body is significantly affected by cell oxidative status and actions of taurine on other aspects of the immune system.

superoxide dismutase (Figure 14.2). The generation of H_2O_2 is converted to hypochlorous acid by the halide-dependent myeloperoxidase pathway. After the state of oxidative stress that destroys the pathogens, neutrophils undergo apoptosis and finally death. Taurine reacts to hypochlorous acid to form taurine chloramine by Cl^- binding to the N-terminal of taurine. Taurine chloramine is an oxidant but less oxidative and more stable than hypochlorous acid. Thus, taurine chloramine increases the phagocytic activity of neutrophils and delays the apoptosis. Taurine chloramine released from activated neutrophils following their apoptosis inhibits the production of inflammatory mediators, such as superoxide anion, nitric oxide, TNF-α, ILs, and prostaglandins in inflammatory cells (e.g. macrophages) at inflammatory tissues (Kim and Cha, 2014).

Taurine chloramine rather than taurine itself plays a key role in immune function. For example, WF10 (a chlorite-based drug) induces endogenous taurine chloramine in peripheral blood mononuclear cells (monocyte, T and B lymphocytes) *in vitro* (Giese *et al.*, 2004). Proliferation and IL-2 production of anti-CD3 stimulated peripheral blood mononuclear cells are inhibited by WF10, as is the nuclear factor of activated T-cells. However, in peripheral blood mononuclear cells and monocytes, WF10 induces pro-inflammatory cytokines like IL-1β, IL-8, and TNF-α. In the monocytic cell line THP-1, WF10 activates activator protein 1 and nuclear factor kappa-light-chain-enhancer of activated B cells. Inhibition of nuclear factor of activated T-cells regulated genes in activated lymphocytes in concert with the induction of several myeloid cell associated pro-inflammatory genes in monocytes represents a novel mechanism of immune modulation. Adding taurine chloramine to the leucocyte cultures also yields responses similar to those of WF10 (Kim and Cha, 2014).

Taurine chloramine is generated primarily by neutrophils and monocytes. Human peripheral blood monocytes, incubated with opsonized zymosan particles, lyse human erythrocyte targets (Dallegri *et al.*, 1988). Cells derived *in vitro* from monocyte-derived macrophages fail to lyse in this way, but when added to the monocyte-red blood cell system, monocyte-derived macrophages enhance lysis. The lysis by monocytes and monocytes plus monocyte-derived macrophages is prevented by taurine, consistent with the requirement for hypochlorous acid. Monocyte-derived macrophages cannot generate hypochlorous acid but augment the hypochlorous acid recovery from the monocyte-red blood cell system. In addition, monocyte-derived macrophages may enhance the polymorphonuclear cell-mediated lysis by improving the hypochlorous acid production, presumably by supplying extra amounts of H_2O_2 to be handled by the neutrophil myeloperoxidase system (Dallegri *et al.*, 1987).

In addition, taurine chloramine generated by neutrophils may transport into macrophages and thus activate phagocytosis. In macrophages, taurine chloramine increases the expression of antioxidant proteins, such as heme oxygenase 1, peroxiredoxin, thioredoxin, glutathione peroxidase, and catalase (Kim and Cha, 2014). Thus, a central role of taurine chloramine is to trigger the resolution of inflammation and protect macrophages and surrounding tissues from being damaged by cytotoxic reactive oxygen metabolites that are overproduced during inflammation.

14.6 Taurine in innate immunity

Neutrophil extracellular traps comprise extracellular chromatin and granule protein complexes that immobilize and kill bacteria, and taurine can decrease neutrophil extracellular trap release. The neutrophil extracellular trap release represents a recently discovered, novel anti-microbial strategy, regulated non-exclusively by NADPH oxidase generation of reactive oxygen intermediates, particularly H_2O_2 *in vitro*. Neutrophil extracellular trap release by human peripheral blood neutrophils treated with exogenous superoxide dismutase supports the link between H_2O_2 and neutrophil extracellular trap production (Palmer *et al.*, 2012). However, treatment with myeloperoxidase inhibitors and direct addition of hypochlorous acid also indicates the significant and sufficient role of hypochlorous acid for neutrophil extracellular trap release. This is confirmed by the ability of hypochlorous acid to stimulate neutrophil extracellular trap release in neutrophils of chronic granulomatous disease patients, which lack a functional NADPH oxidase and consequently neutrophil extracellular trap release in response to classical stimuli. Taurine supplementation decreases neutrophil extracellular trap release *in vitro* due to taurine's conversion of hypochlorous acid to taurine chloramine and stimulation of myeloperoxidase. Note, O_2, H_2O_2, and hypochlorous acid are subsequently reduced in neutrophils (Muhling *et al.*, 2005). Thus, taurine is an anti-oxidant and decreases the neutrophil extracellular trap release from viable neutrophils. Although taurine chloramine possesses oxidative activity, its oxidative capacity is much less than that of H_2O_2 and hypochlorous acid, and neutrophil extracellular trap production can be observed in case of neutrophil death; suggesting that taurine and/or taurine chloramine has an anti-apoptotic activity and can delay cell death.

Taurine chloramine is known as an anti-microbial and anti-inflammatory long-lived oxidant *in vitro*, taurine chloramine inhibits the production of pro-inflammatory cytokines (IL-6 and IL-8) by rheumatoid arthritis synoviocytes. Thus, taurine chloramine treatment delays the onset of collagen-induced arthritis, but does not affect the severity of arthritis (Kwasny-Krochin *et al.*, 2002). Taurine chloramine inhibits proinflammatory mediators such as nitric oxide, TNF-α, and prostaglandin E_2 in activated rodent macrophages. Taurine chloramine suppresses superoxide anion, IL-6, and IL-8 production in activated human polymorphonuclear leucocytes separated from peripheral blood. Further, in human peripheral blood mononuclear leucocytes, taurine chloramine suppresses lymphocyte proliferation (Park *et al.*, 2002). Production of IL-2, IL-6, and IL-8 in phytohemagglutinin-activated non-adherent leucocytes is also inhibited, while that of IL-1β, IL-6, and IL-8 is decreased in lipopolysaccharide-activated adherent monocytes by taurine chloramine.

Dendritic cells play a major role at the initial phase of immune response in which they phagocytize the pathogen products and cause the release of several cytokines. In $N418^+$, major histocompatibility complex class II^+, $B7\text{-}2^+$ dendritic cells generated from the mouse bone marrow cells cultured in the presence of granulocyte-macrophage colony-stimulating factor, taurine chloramine inhibits the production of nitric oxide, reactive oxygen species, IL-6, TNF-α, and IL-12 by interferon-γ and lipopolysaccharide stimulation in a dose-dependent manner (Marcinkiewicz *et al.*, 1999). In addition, taurine chloramine selectively modulates the ability of

dendritic cells to induce the release of IL-2 and IL-10 from T lymphocytes. As mentioned earlier, neutrophils are the major source of taurine chloramine in the tissue, suggesting that the anti-inflammatory role of taurine chloramine from neutrophils is related to modulation of dendritic cells, macrophages, and T lymphocytes at the inflammatory site.

14.7 Taurine in cell-mediated immunity

Taurine also plays a role in proliferative responses to the co-stimulation with phorbol myristate acetate and suboptimal doses of ionomycin in the purified T and B cells from old mice are lower than those from young mice (Nishio *et al.*, 1990). The age-related decline is more significant in T than B lymphocyte. Although taurine increases the proliferative responses of T cells from both young and old mice, the old mice display a greater T cell response. Without taurine, stimulation with phorbol myristate acetate and ionomycin causes decreased intracellular free calcium ion levels in the T cells from old than young mice. In the presence of taurine, the intracellular free calcium ion level increases more in the T cells from old than young mice. This indicates that taurine is important for B and T lymphocyte production by the mechanism related to taurine-regulated intracellular calcium levels, but the augmentation of lymphocytosis per se may be due to more anti-oxidative effects of taurine than taurine itself. For instance, antioxidants glutathione, N-acetylcysteine, thioproline, and taurine are equally effective in increasing proliferation and mobility of lymphocytes from axillary nodes, spleen, thymus, and peritoneum of young-adult BALB/c mice (De La Fuente *et al.*, 2011).

Taurine chloramine inhibits the generation of macrophage inflammatory mediators such as nitric oxide, prostaglandin E_2, TNF-α, and IL-6. In *in vitro* studies, taurine chloramine treatment inhibits IL-2 release in response to mitogen and antigen stimulation. Further, pretreatment of higher numbers of A-20 antigen presenting cells with taurine chloramine in the presence of ovalbumin enhances subsequent presentation of ovalbumin to a specific T-cell hybridoma (Marcinkiewicz *et al.*, 1998), indicating that taurine chloramine may link together innate and adaptive immunity by modulating antigen-presenting processes.

T-cell activation and the subsequent transformation of activated T-cells into T-cell blasts require significant changes in cell volume. Taurine is the main organic osmolyte in all mammalian cells including lymphocytes. Maintaining intracellular taurine content is dependent on taurine transporters. Taurine transporter knockout mice display low intracellular taurine levels, and T-cell mediated recall responses are severely impaired in taut-/- mice (Kaesler *et al.*, 2012). $CD4^+$ and $CD8^+$ T cells are located within peripheral lymph nodes of unprimed taut-/- mice but are decreased in taut-/- compared to taut+/+ mice following *in vivo* activation. Further, in taut-/- mice, antigen challenge reduces $CD4^+$ and $CD8^+$ T cells and thus, impairs *in vivo* generation of T-cell memory. This indicates that taurine transporter and consequently cell taurine content is crucial for T-lymphocyte function.

Taurine plays a key role in both innate and cell-mediate immune responses to reduce tissue damage induced by bacterial infection. In a rat model of mastitis induced by *Streptococcus uberis* infusion into each early postnatal rat mammary glands, inflammation is evidenced by swelling, secretory epithelial cell degeneration, increased adipose tissue, and neutrophil infiltration. Taurine pretreatment attenuates these morphologic changes, the expression of IL-2, interferon-γ mRNA, myeloperoxidase activity, and N-acetyl-β-D-glucosaminidase in mammary tissue (Miao *et al.*, 2012). The increased percentages of forkhead box P3 ($Foxp3^+$), $CD25^+$, $CD4^+$ lymphocytes (regulatory T cells) in mastitis are augmented by taurine pretreatment.

IL-2 immunotherapy used for treatment of metastatic melanoma and renal cell carcinoma mediates its effects through the clonal expansion of lymphocytes. Although IL-2 remains the most effective form of therapy for these cancers, response rates are poor and dose escalation is counter indicated due to side effects including vascular leak and lymphopenia. The mechanism underlying T-cell loss is proposed to be the induction of activation-induced cell death mediated by FasL. In *in vitro* studies, IL-2-treated anti-CD3-activated Jurkat T cells increase FasL expression and apoptosis (Maher *et al.*, 2005). Taurine pretreatment down-regulates FasL protein expression and partially inhibits apoptosis. Inhibition of FasL-signaling also decreases the apoptosis of T cells. Further, stimulation of $CD4^+$ circulating T cells induces apoptosis in sensitized, but not freshly isolated T cells, and this is partially inhibited by taurine. In addition, taurine down-regulates FasL protein expression related to decreased FasL mRNA expression and reduced nuclear factor kappa-light-chain-enhancer of activated B cells activation. Thus, taurine may reverse the lymphopenia associated with IL-2 therapy by suppressing FasL protein production in T lymphocytes.

Cell shrinkage appears to be an important apoptotic process to induce cell death that involves taurine action. Jurkat human T lymphocytes apoptosis is induced by triggering the Fas(CD95) receptor with monoclonal crosslinking antibody. Triggering the Fas(CD95) receptor induces cellular taurine release that coincides with cell shrinkage (Lang *et al.*, 1998). In contrast, pretreatment of cells with Fas(CD95) antibody induces rapid taurine release, cell shrinkage, and DNA fragmentation, indicating that Fas(CD95) receptors play a key role in apoptotic processes via regulation of taurine release in human T lymphocytes. Thus, taurine plays a significant role in immune system starting from the initial step of pathogen-leucocyte interaction to the end of apoptotic cell death of T lymphocytes.

14.8 Taurine in humoral immunity

In 1992, Porter and Martin reported a specific transport system for taurine in chick B cells. The chick B cells maintain a high intracellular taurine concentration (0.8-1.12 mM) that decreases with age. The B cells exhibit carrier-mediated and simple diffusion uptake components, but only the carrier-mediated component increases with age. Further, B-cell taurine uptake is sodium and energy dependent. It is well-known that taurine regulates cell calcium transport. In chick B cells, the calcium uptake rate decreases with age, which is exacerbated by taurine treatment. In

contrast, (Ca^{2+}+Mg^{2+})-ATPase activity increases with age and is stimulated by taurine (Porter and Martin, 1993).

Lines of evidence indicate that taurine and/or taurine chloramine regulates apoptotic cell death in both T and B lymphocytes. Murine pro-B-cell lymphoid (FL5.12) cells are lymphocytic cells that undergo apoptosis following IL-3 withdrawal. Taurine chloramine treatment activates a cell death pathway with kinetics very similar to IL-3 withdrawal (Emerson *et al.*, 2005). Taurine chloramine-treated cells undergo an annexin V-positive/propidium iodide-negative death consistent with apoptosis. Both Bcl-2 and the dominant negative form of caspase-9 inhibit cell death following taurine chloramine treatment. In contrast to IL-3 withdrawal, taurine chloramine treatment of FL5.12 cells induces a rapid cell cycle arrest independent of cell cycle phase. Together, this indicates that taurine chloramine regulates apoptosis in B cells by accelerating cell cycle-independent proliferative arrest followed by the activation of a cell death pathway involving Bcl-2 family members and caspase activation.

14.9 Taurine in blood coagulation

Platelets rich in taurine reduce platelet aggregation. Compared to omnivores, vegans (who are usually depleted of taurine) are more sensitive to pro-aggregating agonists (McCarty, 2004a). The effect of taurine on blood coagulation is complex and depends on doses of taurine, duration of treatment, and subject variability. Taurine supplementation decreases platelet aggregation in both normal and high fat-fed Sprague-Dawley rats (Park *et al.*, 2007). Interestingly, in rats, taurine supplementation decreases aggregation, but in humans, the situation is more complex. In overweight pre-diabetic men, receiving a daily 1.5 g supplementation of taurine for eight weeks does not affect platelet aggregation (Spohr *et al.*, 2005), and in healthy Japanese people, there is no correlation between urine taurine concentration and global thrombosis test-occlusion times (measuring reactivity of platelets) (Ijiri *et al.*, 2013). In contrast, urine taurine concentration is inversely correlated to global thrombosis/lysis times, i.e. spontaneous thrombolytic activity.

Rupture of atherosclerotic plaque triggers the majority of myocardial infarctions. The rupture is activated by matrix metalloproteases secreted by intimal macrophages and foam cells (McCarty, 2004b), and the activation of matrix metalloproteases is mediated, in large part, by phagocyte-derived hypochlorous acid. As mentioned earlier, taurine converts hypochlorous acid to taurine chloramine, a lesser oxidative but more stable oxidant than hypochlorous acid (Ogino *et al.*, 2009). Thus, taurine supplementation may be potentially useful to prevent sudden rupture of atherosclerotic plaque, while slowly increasing thrombolysis.

We previously reported that perinatal taurine supplementation induces thrombocytopenia and increases mean platelet volume (Vijitjaroen *et al.*, 2015). Thrombocytopenia may occur due to an imbalance of platelet production and destruction, and a larger platelet volume commonly reflects increased platelet production (Gladwin and Martin, 1990; Threatte, 1993). It is likely that the low platelet level in our study resulted from an increase in platelet destruction, rather

than a decreased production. This is supported by the observed rise in red blood cell count, hematocrit, normal red blood cell size, and normal hemoglobin content in adult rats perinatally supplemented with taurine. That is, low plasma platelet concentration stimulates bone marrow to increase cell production leading to increases in new platelets. Since these rats receive enough taurine from rat chow after weaning, the abnormal platelet count and volume in adult offspring is programed early in life by perinatal taurine supplementation.

14.10 Therapeutic applications of taurine and taurine derivatives

In growing Japanese quail (1 to 42 days old), dietary 0.05% taurine improves feeding efficiency but decreases food intake (Wang *et al.*, 2009a). The relative weights of thymus are greater in the quails given 0.01% taurine in diet. The relative weights of the bursa of Fabricius and thymus are greater in dietary 0.05% taurine compared to the zero taurine control. The response to phytohemagglutinin is increased by 0.05% taurine supplementation. Therefore, the taurine-supplemented diet has a beneficial effect on immune responses and performance in growing Japanese quail.

Taurine supplementation is recommended for patients with severe depression. Depression is a serious disease involving immune dysfunction, and taurine supplementation is known to improve neural function and immunity. As mentioned earlier, taurine levels in leucocytes affect their immune function. Taurine, aspartate, and glutamine levels are increased in the lymphocytes of depressed (vs. control) patients prior to mirtazapine treatment but are normalized after treatment (Lima *et al.*, 2003). Lymphocytic γ-aminobutyric acid and glutamate levels do not differ between patients and controls. Before mirtazapine treatment, the severity of the disorder is positively correlated with the taurine level in the lymphocytes of depressed patients. This correlation is abolished after treatment. This indicates an abnormal taurine content in lymphocytes of depressive patients that may underlie immune dysfunction in these patients.

The efficacy of methotrexate, a widely used cytotoxic chemotherapeutic agent, is often limited by severe side effects and toxicity. Methotrexate administration increases the malondialdehyde, myeloperoxidase activity, and collagen contents, while decreases glutathione levels in all rat tissues, and these alterations are reversed by taurine treatment (Cetiner *et al.*, 2005). Oxidative burst of neutrophils stimulated by phorbol myristate acetate is reduced following saline-treatment with methotrexate, and taurine abolishes this effect. Further, leucocyte apoptosis and cell death are increased in methotrexate-treated animals, while taurine treatment reverses these effects. The reduced cellularity in bone marrow in methotrexate-treated groups is reversed back to control levels in taurine-treated rats. Severe degeneration of the intestinal mucosa, liver parenchyma, glomerular, and tubular epithelium observed in saline-treated methotrexate group is improved by taurine treatment. Thus, taurine supplementation protects against methotrexate-induced oxidant organ damage and inhibits leucocyte apoptosis resulting from chemotherapy.

The enhancing effect of taurine on the function of leucocytes after chemotherapy is also reported in lung cancer (Wang *et al.*, 2009b). Lewis lung carcinoma-bearing mice were given taurine

combined with cyclophosphamide. The tumor inhibition rates on Lewis lung carcinoma in taurine combined with cyclophosphamide are higher than that in the cyclophosphamide alone. Compared to cyclophosphamide alone, taurine plus cyclophosphamide treatment increases bone marrow nucleate cells and white blood cells, spleen index, thymus index, lymphocyte proliferation, phagocytic activity of peritoneal macrophage, peripheral blood neutrophils, and monocytes. These lines of evidence suggest that taurine supplementation prevents several hematologic and immune side effects induced by chemotherapy.

14.11 Concluding remarks

White blood cells are rich in taurine, which plays many physiologic and pharmacologic functions in the human body including the immune system. Taurine and its derivative taurine chloramine play crucial roles in hematopoiesis of leucocytes and platelets, T- and B-cell differentiation and maturation, phagocytic cells activity, innate and adaptive immune function, and complement system activity. Taurine functions to ensure that the immune cells are produced at a sufficient amount and rate on demand, survive to play their optimal functions, and then undergo adaptive apoptosis. For hematologic function, taurine actions involve cell proliferation, cell volume regulation, anti-oxidation, cytokine release, and anti-apoptosis in white blood cells, while in optimal platelets, taurine levels are necessary for clotting formation and lysis balance to prevent thrombosis. Thus, taurine content is observed in healthy food from energy drinks to supplement diets. Clinically, taurine supplementation prevents hematologic side effects of chemotherapy in cancer patients. In addition, taurine regulates early life programing that influences adult hematologic properties. These lines of evidence indicate the important role of taurine throughout life. However, the beneficial and clinical significances of taurine are mostly studied in animal and *in vitro* experiments. Therefore, clinical applications of taurine therapy in patients with hematologic disorders are limited and require rigorous study if the animal and *in vitro* studies are to be translated from bench to bedside.

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15. L-carnitine in uremic anemia

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Abstract

Anemia, defined as a qualitative or quantitative deficiency of hemoglobin, is a frequent and serious complication of chronic kidney disease (CKD). Deficient erythropoietin synthesis, iron deficiency, blood loss, and a decreased erythrocyte half-life are the main contributors to anemia associated with CKD. Anemia in CKD patients contributes to many of the symptoms associated with the disease, including fatigue, depression, reduced tolerance of exercise, dyspnea, cardiovascular consequences such as left ventricular hypertrophy and left ventricular systolic dysfunction, and increased risk of mortality. The introduction of erythropoietin analogues, also termed erythropoiesis stimulating agents (ESAs), has revolutionized the care of renal anemia patients. Today ESAs and iron therapy are the main tools for treating anemia associated with CKD. The available ESAs are generally very effective drugs, and real resistance to their action is rarely seen. However, several factors can cause incomplete response to ESA: recognition and correction of these may improve anemia management. If there are no correctable underlying causes or deficiency states limiting erythropoiesis, in patients with poor response or needing high ESA doses adjuvant therapies may be employed to increase the hemoglobin level and improve ESA cost/efficacy. Adjuvant therapies for renal anemia include the naturally occurring compound L-carnitine. Here, we here review the evidence available for L-carnitine treatment in dialysis patients suffering from uremic anemia.

Keywords: chronic kidney disease, erythropoietin, erythropoiesis stimulating agent, hemodialysis, red blood cell

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Key facts

- Normochromic normocytic anemia is a frequent and serious complication of chronic kidney disease. It reduces the quality of life and has been associated with an increased mortality risk.
- Deficiency of erythropoietin, a glycoprotein hormone produced by the kidney that controls erythropoiesis, is the main cause of anemia in chronic kidney disease. Introduction of human recombinant erythropoietin has had a fundamental role in the management of anemia in patients with chronic kidney disease.
- Patients who undergo chronic hemodialysis usually present carnitine insuf ciency: clinical consequences may be: impaired muscle function, decreased wound healing, altered ventilatory response, and abnormal immune function.
- Carnitine supplementation can improve the nutritional status of hemodialysis patients promoting a positive protein balance, alleviate muscle discomfort, and also ameliorate erythropoietin-resistant anemia.
- A large number of studies have examined the role of L-carnitine supplementation in uremic anemia treatment, but the results are not uniform. Well-designed studies are required in the future to definitively establish the therapeutic role of L-carnitine in anemia of chronic kidney disease.

Summary points

- Anemia is a major clinical manifestation in nephropathic patients.
- The introduction of human recombinant erythropoietin in clinical practice has radically changed the treatment of uremic anemia, proving to be the most effective therapeutic tool.
- There are however several causes of hypo-responsiveness to erythropoiesis stimulating agents in chronic kidney disease patients.
- Carnitine is an important intermediary in fat metabolism and is crucial for energy production in tissues dependent upon fatty acid oxidation.
- Regular carnitine supplementation in patients on chronic hemodialysis can improve lipid metabolism, protein nutrition, antioxidant status, and anemia.
- L-carnitine has biophysical, metabolic and antiapoptotic effects on erythropoiesis and the function of mature, circulating erythrocytes.
- The therapeutic role of L-carnitine in the anemia of ESRD is still debated. Larger well-designed studies are necessary to better define the clinical implications of LC supplementation in uremic anemia treatment.

Abbreviations

ACS	Acyl-CoA synthetase
CKD	Chronic kidney disease
CoA	Coenzyme A
CoA-SH	Reduced coenzyme A
CPT	Carnitine palmitoyltransferase 1
EPO	Erythropoietin
ESA	Erythropoiesis stimulating agent
ESRD	End-stage renal disease
Hb	Hemoglobin
Hct	Hematocrit
HD	Hemodialysis
IV	Intravenous
LC	L-carnitine
LPLAT	Lysophospholipid acyltransferase
PLA_2	Phospholipase A_2
PLP	Phospholipid
RBC	Red blood cell
rHuEPO	Human recombinant erythropoietin
ROS	Reactive oxygen species

15.1 Uremic anemia

Anemia is an important clinical sign present in nearly all patients with CKD, which contributes considerably to these patients' reduced quality of life and has been associated with a number of adverse clinical outcomes including increased mortality risk. Renal anemia is typically an isolated normochromic, normocytic anemia without thrombocytopenia or leucopenia. Though the Hct may vary considerably in patients with a comparable reduction in kidney function, except for polycystic kidney disease the underlying primary renal disease appears to have no specific effect on the degree of anemia. The main cause of CKD anemia is a reduction in erythropoiesis due mainly, though not exclusively, to inadequate production of EPO by the failing kidney. EPO is normally produced by interstitial fibroblasts in the renal cortex, in close proximity to tubular epithelial cells and peritubular capillaries (Eckardt, 1994; Jelkmann, 2004). EPO is a glycoprotein hormone consisting of a 165-amino acid protein backbone and four complexes, heavily sialylated carbohydrate chains (Eckardt, 1994; Jelkmann, 2004). The natural EPO hormone acts by binding to its receptor which is present on erythroid precursors in the bone marrow, thereby activating a complex series of intracellular signals that stimulate erythropoiesis. Upon persisting contact with the receptor, the EPO molecule is internalized and degraded, which represents the primary way of eliminating the hormone from the circulation. Inhibition of red cell production by uremic inhibitors of erythropoiesis may also contribute to the pathogenesis of renal anemia and dialysis

per se can improve renal anemia and the efficacy of ESA (Bonomini and Sirolli, 2003). A further cause of anemia in renal failure is the decreased survival of circulating RBCs.

Before rHuEPO became available, patients on dialysis very often required blood transfusions exposing them to the risk of infection and iron overload; the availability of rHuEPO in the late 1980s changed this situation completely (Table 15.1).

The clinical use of rHuEPO has radically changed the treatment of uremic anemia, proving to be the most effective therapeutic tool. Its use has in fact:

- reduced cardiovascular morbidity and mortality in patients with CKD;
- resulted in an improved quality of life, increasing the feeling of general well-being and resistance to physical stress;
- got round the use of recurrent blood transfusion therapy, previously the main therapeutic option.

rHuEPO (epoetin α and epoetin β) were the first-generation ESAs for use in clinical practice. Subsequently, two second-generation ESAs with a longer duration of action were developed – the hyperglycosylated version of epoetin α, darbepoetin α and a pegylated version of epoetin β, methoxy-polyethylene glycol-epoetin beta. Biosimilar epoetins, together with epoetin theta, have also received marketing authorization in many countries (Jelkmann, 2015).

According to recent guidelines (Kidney Disease: Improving Global Outcomes (KDIGO) Anemia Work Group, 2012), ESA therapy not should be initiated in adult CKD patients with Hb >10 g/dl, because of the risk of stroke; the target Hb concentration should be 11 to 12 g/dl, and should not intentionally exceed 13 g/dl. ESA therapy should be individualized with Hb >10 g/dl, based on the rate of fall in Hb concentration, response to iron therapy, and presence of symptoms related to anemia.

Table 15.1. Milestones in the development of erythropoietin (Singh *et al.*, 2009).

1878	Bert and Jourdaner suggest that symptoms of anemia result from hypoxia
1906	Carnot and Deflandre demonstrate that serum from an anemic rabbit donor injected into normal rabbits results in increased erythropoiesis
1953	Erslev shows how plasma from anemic rabbits contains a factor that stimulates erythropoiesis
1977	Goldwasser's laboratory purifies erythropoietin from large quantities of urine obtained from patients with aplastic anemia
1985	Erythropoietin gene cloned by Lin working at Amgen
1989	Eschbach publishes study in New England Journal of Medicine demonstrating ef cacy of erythropoietin in the treatment of chronic kidney disease anemia
1989	Food and Drug Administration approves the use of erythropoietin in the treatment of anemia in chronic renal failure

Treatment with ESA is most successful in the presence of adequate iron stores. KDIGO guidelines recommend a serum ferritin level >200 μg/ml for HD patients, >100 μg/ml for CKD or peritoneal dialysis patients, and a target % transferrin saturation >20%. Iron may be administered orally or parenterally, although in a large proportion of cases oral supplementation is not effective. Supplementation of IV iron, as compared to the oral route, is associated in HD patients with significantly higher Hb values regardless of the type of formulation and the possible use of ESA (Rozen-Zvi *et al.*, 2008); this superiority was also found in patients with CKD in conservative therapy, although to a lesser extent (Rozen-Zvi *et al.*, 2008). Among the formulations of injectable iron, iron dextran is not recommended due to the risk of severe acute reactions; iron sucrose appears to be the most secure formulation, followed by iron gluconate.

ESA therapy is generally well-tolerated, and adverse effects are uncommon. These may include worsening hypertension, seizures, impaired solute clearance (particularly potassium), and an increased frequency of thrombotic events at, but not confined to, vascular access sites.

Today, ESAs and iron therapy are the primary treatment for anemia in CKD. Real resistance to the action of ESAs is rarely observed. However, many factors can cause an inadequate response to treatment with such agents (Table 15.2).

The recognition and correction (if possible) of such factors can improve anemia and allow ESA to act effectively, even at lower doses. When no correctable underlying causes are detected, and in the absence of deficiency states limiting erythropoiesis, ESRD patients with poor ESA response or needing high doses may be treated with so-called adjuvant therapies. The aim of adjuvant therapy is to increase the Hb level and improve the cost/benefit ratio of ESA. Adjuvant therapies include anti-oxidant agents (glutathione, vitamin E), vitamin C, pentoxyfilline, and changes in the dialytic treatment (convective therapies, ultrapure dialysate).

Table 15.2. Causes of hypo-responsiveness to erythropoiesis stimulating agent therapy in end-stage renal disease patients.

Most common	inadequate dosage
	iron deficiency
	inflammation/infection
Less frequent	deficiency of vitamin B12 or folate
	hyperparathyroidism
	aluminum accumulation
	primitive bone marrow disorders
	hemoglobinopathies
	chronic blood loss, hemolysis
	malnutrition
	drugs (cytotoxics, ACE-inhibitors)

LC, a naturally occurring compound, has also been used as an adjuvant therapy. We here review the evidence for LC use in anemic ESRD patients.

15.2 L-carnitine

15.2.1 Carnitine physiology

Carnitine (β-hydroxy-γ-trimethylaminobutyric acid), a water-soluble zwitterion, plays a number of important intracellular functions (Arduini *et al.*, 2008; Steiber *et al.*, 2004; Zammit, 1999). The most popular and well known role of carnitine is to facilitate the transfer of long-chain fatty acids such as acylcarnitine esters across the inner mitochondrial membrane, thereby acting as an essential cofactor for mitochondrial fatty acid oxidation. However, carnitine enables branched-chain a-keto acid oxidation, shuttles acyl moieties chain-shortened by β-oxidation out of peroxisomes in the liver, and modulates the intramitochondrial acyl-CoA/CoA ratio in mammalian cells. Carnitine also traps potentially toxic acyl-CoA metabolites that may increase excessively during acute metabolic crises through esterification (Steiber *et al.*, 2004). These metabolites may impair the citric acid cycle, gluconeogenesis, the urea cycle and fatty acid oxidation. All these functions center upon one basic, enzyme-catalyzed reaction:

$$\text{carnitine} + \text{acyl-CoA} \rightleftharpoons \text{acyl-carnitine} + \text{CoA-SH}$$

In this freely reversible reaction, the acyl unit can be transferred from CoA to carnitine and vice versa by carnitine acyltransferases, a family of enzymes that are widely distributed in the cell (Ramsay and Naismith, 2003; Zammit *et al.*, 2009). Indeed, the cell relies on such acyltransferases and carnitine to regulate the highly compartmentalized, limited pools of CoA derivatives. Acyl-CoA pools provide activated substrates for many key metabolic pathways such as the tricarboxylic acid cycle and lipid and cholesterol biosynthesis, for posttranslational modification of proteins and for detoxification mechanisms (Zammit, 1999). The reversible transfer of activated acyl groups from the limited pools of membrane-impermeable CoA to the abundant, mobile carnitine provides transport between the various compartments, a considerable reservoir of activated acyl groups and a route for excretion of excess acyl moieties. Transport is required to import fatty acids for energy production in mitochondria (Bremer, 1983) and in yeast peroxisomes (Visser *et al.*, 2007). Carnitine also serves as a reservoir in the form of acetyl-L-carnitine in the heart and sperm (Bremer, 1983) and as long-chain acyl-carnitine esters used for membrane repair when cells lack energy to activate fatty acids (Ramsay and Arduini, 1993). Carnitine derivatives are excreted in the urine and in the bile (Rashed *et al.*, 1995). In general, the carnitine system both connects the different acyl-CoA pools and limits wide oscillations in their acylation state that could be harmful to cellular homeostasis (Zammit *et al.*, 2009). In this regard, the latest thinking on control of autophagy, a process believed to play a key role in ageing, is based on the global protein acetylation status, where acetyl-CoA acts as the main substrate for this modification (Pietrocola *et al.*, 2015).

In omnivores, approximately 75% of carnitine sources come from the diet and 25% from endogenous synthesis (Rebouche, 1992). Major dietary sources of carnitine are meat, poultry, fish and dairy products (Rebouche and Engel, 1984), whereas, in strict vegetarians, endogenous carnitine synthesis provides more than 90% of total available carnitine (Rebouche, 1992). Human milk and most milk-based formulas contain adequate carnitine to sustain early growth and development. In term infants fed with soy protein-based formulas with no carnitine supplementation, plasma carnitine concentrations were markedly lower than in carnitine-supplemented infants (Novak, 1990), though no deficits in growth or development were identified in term infants consuming essentially carnitine-free formulas over at least the first four months of life (Olson *et al.*, 1989). Skeletal muscle contains over 90% of total body carnitine (Rebouche, 1992). The plasma carnitine concentration is largely regulated by the renal threshold, which is approximately 40 mmol/l (Engel *et al.*, 1981). Active transport of carnitine across the proximal renal tubule limits urinary loss. At physiological concentrations, more than 90% of filtered carnitine is reabsorbed by the kidney (Rebouche and Mack, 1984). Urinary loss and growth requirements delimit the need for carnitine, since it does not undergo significant metabolic degradation (De Vivo and Tein, 1990). Human skeletal muscle, heart, liver, kidney and brain are capable of biosynthesizing carnitine from methionine and lysine to its immediate precursor γ-butyrobetaine (Vaz and Wanders, 2002). Final conversion of γ-butyrobetaine to LC by γ-butyrobetaine hydroxylase only occurs in the liver, kidney and brain in humans. γ-butyrobetaine is exported to these tissues for final conversion to LC, which can then be taken up by the rest of tissues. Hepatic γ-butyrobetaine hydroxylase is developmentally regulated, its rate at birth being about 25% of adult activity (Olson and Rebouche, 1987).

15.2.2 Carnitine in hemodialysis and kidney disorders

In healthy individuals, carnitine is filtered and tubular resorption of free carnitine is almost complete. What is excreted in the urine is carnitine ester, or acyl-carnitine (Ahmad, 2001). In healthy people, the renal clearance of acyl-carnitine is four to eight times that of free carnitine (Berard *et al.*, 1995; Wanner *et al.*, 1987, 1988). Impairment of acyl-carnitine excretion occurs with deteriorating renal function leading to decreased carnitine clearance and resulting in elevated plasma levels of carnitine (Ahmad, 2001). Uremic patients have elevated levels of acyl-carnitine that occur as both elevated free carnitine and total carnitine before dialysis (Wanner *et al.*, 1987) These patients experience accumulation of plasma acyl-carnitines, in part due to decreased renal clearance of esterified carnitine moieties (Brass *et al.*, 2001). Due to accumulation of metabolic intermediates, impaired carnitine biosynthesis, reduced protein intake, and increased removal of carnitine through HD, patients who undergo routine HD usually present with plasma carnitine insufficiency (Evans and Fornasini, 2003). During dialysis, patients experience subnormal plasma/serum free carnitine concentrations (Ahmad, 2001) with plasma levels dropping by 60%, there being a slow replacement from carnitine stores such as skeletal muscle during the interdialytic period.

Dietary intake also plays an important role in carnitine homeostasis of HD patients since the prevalence of malnutrition ranges from 18 to 75% of these cases (Mehrotra and Kopple, 2003).

Clinical consequences of such malnutrition can lead to impaired muscle function, decreased wound healing, altered ventilatory response, and abnormal immune function (Calvani *et al.*, 2004). Repeated hemodialytic treatments can result in depletion of skeletal muscle carnitine stores. IV LC following dialysis can replenish the free carnitine removed from the blood and restore muscle carnitine content, alleviating muscle myopathies and impaired exercise capacity (Ahmad, 2001), as well as ameliorating EPO-resistant anemia, decreased cardiac performance and intradialytic hypotension (Brass *et al.*, 2001). Furthermore, carnitine may benefit the nutritional status of HD patients by promoting a positive protein balance, and by reducing insulin resistance and chronic inflammation, possibly through an effect on leptin resistance (Calvani *et al.*, 2004). Handelman, however, cautions that evidence for the effectiveness of carnitine supplements in dialysis suffers from trials being limited in subject number and open labeled, and suggests that more rigorous testing is needed (Handelman, 2006).

15.2.3 Carnitine in circulating red blood cells

As discussed above, the majority of carnitine is confined within skeletal muscle and the rest of it may be found in organs such as the liver, brain and kidney (Zammit, 1999). Small amounts of carnitine may also be found in circulating RBCs, though it was believed that its presence therein was somehow a vestigial remnant of the 'previous' life of the erythrocyte as a nucleated, mitochondrion enriched precursor: the reticulocyte. In keeping with this observation, RBCs also contain CPT, a key enzyme involved in the transfer of long-chain acyl units across the outer mitochondrial membrane (Zammit, 1999). Here too, the belief was that such CPT was part of the 'previous' life of RBCs. This concept was further reinforced by the dominant mitochondriocentric view of the carnitine system, which ruled out *a priori* any physiological role for the carnitine system in mature RBCs (Bremer, 1983). As we will be present and discuss below, not only does the carnitine system have a role in RBC membrane physiology, but it also sheds some light on the mechanism behind the therapeutic intervention of carnitine on uremic anemia.

PLPs are the main constituents of biological membranes, playing key roles in maintaining the cellular boundary, regulating cellular signaling, and preserving membrane fluidity and cytoskeleton stability (Doherty and McMahon, 2008; Evans *et al.*, 1976; Mohandas and Gallagher, 2008). In addition, these last attributes allow RBC to tolerate a variety of chemical and physical challenges during their lifespan (i.e. they can deform their resting diameter from about 8 μm to the diameter of the capillary bed and splenic sinusoids that are smaller than 1.5 μm). Kennedy and Lands reported the synthesis of PLP in the *de novo* pathway (Kennedy pathway), and the maturation of PLPs in the remodeling pathway (Lands cycle), respectively, to produce membrane diversity (Kennedy and Weiss, 1956; Lands, 1965; Waku and Lands, 1968). Mature RBCs contain no intracellular organelles and, unlike the brain and other organs, lack the metabolic machinery for *de novo* fatty acid synthesis, fatty acid desaturation and elongation, or PLP and triglyceride synthesis. The fatty acid composition of RBC membrane PLPs, however, is not fixed during the lifetime of RBCs. Indeed, several studies have shown that the dietary fat composition affects the fatty acid composition of RBC membrane PLPs (Abbot *et al.*, 2012; Hodson *et al.*, 2014, 2008; Peet *et al.*, 2004). The turnover of RBC fatty acids esterified in membrane PLPs occurs by several

mechanisms that differ in relevance to the various PLP classes, and proceeds at different rates for different fatty acids. One main metabolic pathway devoted to the remodeling of fatty acids esterified in RBC membrane PLPs is known as the Lands cycle (Waku and Lands, 1968), a process occurring at the sn-2-position of the glycerol backbone of membrane PLPs through the concerted and coordinated action of PLA_2 and LPLAT. This coordinated deacylation-acylation activity requires a fine rate-coupling between PLA_2 and LPLAT rates, since the availability of acyl-CoA is in turn dependent on a third enzyme known as ACS. Indeed, as shown in Figure 15.1, a more comprehensive view of the Lands cycle, taking into account all the key players of remodeling activity, requires the addition of another cycle: ACS-LPLAT. The resulting 'bicycle', however, may not be sufficient to guarantee an efficient remodeling/re-esterification process capable of responding to potential oscillations in the PLA_2 rate (see also below). In addition, the amount of free CoA-SH available in RBCs is very small, which may become a limiting factor in conditions where the ACS rate is unable to follow or couple with the LPLAT rate. For example, an increased demand upon activated acyl units to reacylate membrane lysophospholipids might not be compensated by an equivalent increase in the LPLAT and/or ACS rates, as seen in the course of an oxidative challenge (Dise and Goodman, 1986; Lubin, 1972). This might easily result in a significant imbalance of the acyl-CoA to free CoA-SH ratio, which in turn may affect LPLAT and/or ACS activities, or even severely limit free CoA-SH availability for the reacylation of membrane lysophospholipids. Again, these processes may also occur in conditions where ATP availability may be the limiting factor, which would further affect the ACS activity in providing activated acyl units for the remodeling process (Arduini *et al.*, 1992). Thus, from the kinetic standpoint the Lands cycle described in Figure 15.1 may be viewed as a 'rate uncoupled' bicycle, a pathway which may not be able to guarantee optimal reacylation/remodeling activity by membrane PLPs. It is worth mentioning that even under saturation conditions, specific ACS and LPLAT activities for

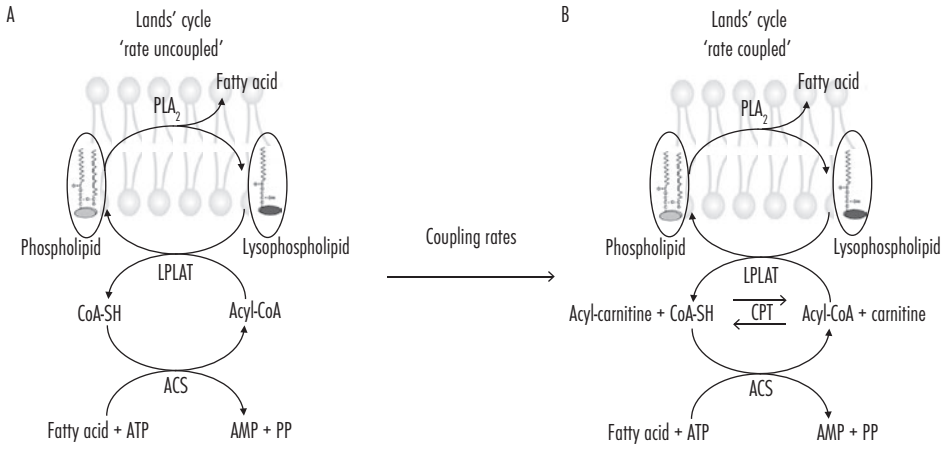

Figure 15.1. Potential metabolic intervention of carnitine and carnitine palmitoyltransferase 1 (CPT) on membrane phospholipid fatty acid turnover. (A) Land's cycle 'rate uncoupled'. (B) Land's cycle 'rate coupled'. ACS: acyl-CoA synthetase; CoA-SH: reduced coenzyme A; LPLAT: lysophospholipid acyltransferase; PLA_2: phospholipase A_2.

a variety of long-chain fatty acids and their respective CoA esters are quite different: the specific activity of ACS with arachidonic acid is ten times higher than that of LPLAT with arachidoyl-CoA and lysophosphatydilcholine (Bakken and Farstad, 1989; Shindou and Shimizu, 2009).

In Figure 15.1A the 'rate uncoupled' Land's cycle is shown. The first half of the originally formulated Land's cycle is represented by the deacylating activity of PLA_2, which results in the hydrolysis of a fatty acid esterified in the second position of the glycerol backbone of membrane phospholipids and the release of the corresponding lysophospholipid. The second half of the cycle is represented by the reacylating activity of LPLAT, which regenerates the native phospholipid, limiting the accumulation of the potentially toxic lysophospholipids. However, since LPLAT activity requires an adequate supply of activated acyl units (acyl-CoA) from the activity of the ATP-dependent enzyme ACS, the combined LPLAT and ACS activities may be seen as an additional cycle adjacent to the original Land's cycle. As the enzymatic rates of the PLA_2, LPLAT and ACS are not necessarily coupled, one may envision the two combined cycles as 'rate uncoupled'.

Figure 15.1B shows the 'rate coupled' Land's cycle. Since the kinetic efficiency of the two cycles is strictly dependent on the extent of the enzymatic rates of PLA_2, LPLAT and ACS, the presence of a rate-coupler between LPLAT and ACS may overcome the risk that significant rate differences between the three enzymatic steps impairs membrane phospholipid fatty acid turnover. Therefore, the addition of a rate-coupler, CPT, to the previous scheme would allow the ACS-LPLAT cycle to keep the acyl-CoA/free CoA ratio constant. Indeed, given the kinetic properties of CPT, any alteration of the acyl-CoA/free CoA ratio would be promptly buffered by the fully reversible and mass action sensitive CPT reaction. The presence of supraphysiological concentration of carnitine would further support the buffering action of CPT.

The inability to respond adequately to potential oscillations in enzymatic rates and their related substrate/product concentrations may be further exacerbated during an oxidative challenge. Because of high levels of oxygen along with iron-containing Hb, RBCs are indeed continuously exposed to ROS (Bonomini *et al.*, 2011). Although the high level of ROS in RBCs is counteracted by an elaborate antioxidant system, which includes proteins such as superoxide dismutase and catalase, thiol species such as glutathione and peroxyredoxin, and vitamin E, damage will occur and repair of oxidized molecules is needed to maintain functional viability of the membrane (Kuypers, 2008; Lang *et al.*, 2014; Mohanty *et al.*, 2014). One well-known damaging action by ROS is the peroxidation of unsaturated fatty acids esterified in membrane PLPs. Hence, to limit the potentially harmful effects of oxidatively-modified fatty acids on the overall stability of RBC membrane, a PLA_2 promptly removes the modified fatty acid, which is replaced by a native one thanks to the reacylating activity of LPLAT (Lubin, 1972). As discussed above, it is clear that under an oxidative challenge the efficiency of the membrane PLP repair process may be severely impaired by a 'rate uncoupled' Lands cycle. To overcome such a kinetic impasse, an additional player is required: CPT (Arduini, 1992; Arduini *et al.*, 1997, 1992; Bonomini *et al.*, 2011; De Los Reyes *et al.*, 1998; Ramsay and Naismith, 2003; Ramsay *et al.*, 1991). As shown in Figure 15.1, introducing the reaction catalyzed by CPT between LPLAT and ACS would make the Lands cycle properly 'rate coupled' and able to buffer significant differences in their rates, which would

translate into a more stable acyl-CoA to free CoA-SH ratio. Now, since the reaction catalyzed by CPT is freely reversible, and thus sensitive to mass-action by the various substrates, an ACS rate higher than LPLAT would be promptly buffered by CPT, pushing the reaction toward acyl-carnitine (Arduini *et al.*, 1992; Ramsay and Arduini, 1993; Zammit, 1999). In this manner, a potential harmful depletion of free CoA-SH may be avoided. Again, under this condition, a reservoir of acyl-unit such as acyl-carnitine may become available at no ATP cost whenever the ACS activity is affected by reduced ATP availability or an increased demand for activated acyl-units (i.e. higher PLA_2 rates) cannot be matched by a parallel increase in ACS rates (Arduini *et al.*, 1997). Several studies have confirmed these concepts in both healthy and pathological RBCs by means of different experimental strategies (Allen and Manning, 1994; Arduini *et al.*, 1997, 1992, 2001; Darghouth *et al.*, 2011; De Los Reyes *et al.*, 1998).

15.3 L-carnitine treatment and uremic anemia

A large number of studies examining the effects of LC on anemia indices (Hb, Hct, ESA dose) have been carried out in patients on chronic HD treatment. Before the introduction of rHuEPO in clinical practice, six trials had been published. Randomized, placebo-controlled studies showed an increase in Hct following LC oral administration, 1.65 g daily for 52 weeks (Trovato *et al.*, 1982) or 2 g daily for two months (Bellinghieri *et al.*, 1983), but no change in Hb after 2 g IV per dialysis session over a six-week period (Nilsson-Ehle *et al.*, 1985). Four months' treatment with LC (20 mg/kg/HD session) followed by four months' placebo-treatment, was associated with increased Hct (Vacha *et al.*, 1983). Finally, two open-label studies with no control group demonstrated an increase in Hb and Hct after two months' LC (2 g daily) oral administration (Donatelli *et al.*, 1987), and an increase in Hct following LC treatment either orally (1 g daily) or in the dialysate (100 mmol/l) over six consecutive months (Albertazzi *et al.*, 1983).

A summary of published studies evaluating the effects of LC treatment on anemia in ESRD patients after ESA became available is reported in Table 15.3 (trials reporting favorable effects) and in Table 15.4 (trials reporting no effects).

A systematic review to determine the effects of LC in maintenance HD patients suggested a promising effect by the compound on anemia management (Hurot *et al.*, 2002). In five of the six identified randomized trials a reduction ($P<0.01$) in EPO dose was achieved in LC-treated patients while maintaining a comparable target Hb in both the carnitine and control groups; the effects of LC supplements between trials were however heterogeneous, somewhat detracting from the conclusion. In addition, the EPO resistance index, defined as the EPO dose given per gram of Hb to maintain a constant Hb level (Kletzmayr *et al.*, 1999), was found to be improved (Hurot *et al.*, 2002). By contrast, a more recent systematic review and meta-analysis did not confirm the above findings (Yang *et al.*, 2014). Nine randomized controlled trials were included in the meta-analysis (Biolo *et al.*, 2008; Brass *et al.*, 2001; Enani Naini *et al.*, 2012; Mitvalli *et al.*, 2005; Rathod *et al.*, 2006; Sabry, 2010; Savica *et al.*, 2005; Steiber *et al.*, 2006; Trovato *et al.*, 1998). The

Table 15.3. Summary of studies showing favorable effects of L-carnitine treatment on anemia indices (hemoglobin, hematocrit, erythropoiesis stimulating agent dose) in adult end-stage renal disease patients on maintenance hemodialysis in the erythropoiesis stimulating agent era.

Study	Study design	Patients (n)[1]	Administration route	L-carnitine dose and regimen[2]	Duration of treatment	Results[3]
Labonia (1995)	randomized, double-blind, placebo-controlled	L=13 C=11	intravenous	1 g/HD treatment	2 months	decreased EPO dose
Boran *et al.* (1996)	open-label, no control group	14	intravenous	40-60 mg/kg weekly	9 months	increased Hct
Kavadias *et al.*, (1996)	open-label, no control group	8	intravenous	2 g/HD treatment	5 months	increased Hct at reduced EPO dose
Trovato *et al.* (1998)	not randomized, open-label	L=25 C=35	intravenous and oral	1 g/HD treatment and 1 g daily	3 years	decreased EPO dose
Nikolaos *et al.* (2000)	open-label, no control group	15	intravenous	30 mg/kg/HD treatment	3 months	increased Hct
Matsumoto *et al.* (2001)	open-label, no control group	14	oral	500 mg daily	3 months	increased Hct
Veselà *et al.* (2001)	not randomized, open-label	L=12 C=12	intravenous	15 mg/kg/HD treatment	6 months	decreased EPO dose
Kadiroglu *et al.* (2005)	not randomized, open-label	L=17 C=17	intravenous	1 g/HD treatment	16 weeks	decreased EPO dose
Mitwalli *et al.* (2005)	randomized, single-blind, placebo-controlled	L=18 C=18[a]	intravenous	15 mg/kg/HD treatment	6 months	increased Hb and Hct
Savica *et al.* (2005)	randomized, placebo-controlled	L=48 C=65	intravenous	20 mg/kg/HD treatment	6 months	increased Hb
Rathod *et al.* (2006)	randomized, single blind, placebo-controlled	L=10 C=10	intravenous	20 mg/kg/HD treatment	8 weeks	increased Hb
Steiber *et al.* (2006)	randomized, double-blind, placebo-controlled	L=25[b] C=25[c]	intravenous	2 g/HD treatment	24 weeks	decreased EPO dose
Enami Naini *et al.* (2012)	randomized, double-blind, placebo-controlled	L=17[d] C=27	oral	1 g daily	4 months	increased Hb

[1] C: control group; L: L-carnitine treated group; [a]: 13 patients completed the study; [b]: 15 patients completed the study; [c]: 19 patients completed the study; [d]: 24 patients completed the study.

[2] HD: hemodialysis.

[3] EPO: erythropoietin; Hb: hemoglobin; Hct: hematocrit.

Table 15.4. Summary of studies showing no effects of L-carnitine treatment on anemia indices (hemoglobin, hematocrit, erythropoiesis stimulating agent dose) in adult end-stage renal disease patients on maintenance hemodialysis in the erythropoiesis stimulating agent era.

Study	Study design	Patients (n)[1]	Administration route	L-carnitine dose and regimen[2]	Duration of treatment	Results[3]
Caruso *et al.* (1998)	randomized, double-blind, placebo-controlled	L=15[a] C=16	intravenous	1 g/HD treatment	6 months	no change in Hct or EPO dose[b]
Kletzmayr *et al.* (1999)	randomized, double-blind, placebo-controlled	L=20[c] L=20	intravenous	5 or 25 mg/kg/ HD treatment	8 months	no change in EPO dose[d]
Vaux *et al.* (2004)	randomized, double-blind, placebo-controlled	L=13 C=13	intravenous	20 mg/kg/HD treatment	16 weeks	no change in Hb or EPO dose
Sabry (2010)	randomized, placebo-controlled	L=20 C=35	oral	1.5 g daily	6 months	no change in Hb or EPO dose
Mercadal *et al.* (2012)	randomized, double-blind, placebo-controlled	L=46[e] C=46[f]	intravenous	1 g/HD treatment	1 year	no change in EPO dose
Kudoh *et al.* (2014)	open-label, no control group	18	oral	900 mg daily	1 year	No change in Hb or EPO dose[g]
Biolo *et al.* (2008)	randomized, double-blind, placebo-controlled	L=9 C=10	intravenous	20 mg/kg/HD treatment	7 consecutive HD sessions	no change in Hb

[1] C: control group; L: L-carnitine treated group. [a]: 12 patients completed the study; [b]: among the 12 evaluable treated patients, EPO dose was reduced in 7; [c]: 19 patients completed the study; [d]: among the 19 evaluable treated patients, EPO dose was reduced in 8; [e]: 35 patients completed the study; [f]: 38 patients completed the study; [g]: among the 18 evaluable treated patients, EPO dose was reduced in 7.

[2] HD: hemodialysis.

[3] EPO: erythropoietin; Hb: hemoglobin; Hct: hematocrit;

result showed no significant (P=0.33) increase in Hb levels in LC-treated groups as compared to groups not receiving LC with evidence of heterogeneity.

Thus, while many studies indicate a favorable effect on uremic anemia following LC administration, particularly in patients not treated with ESA, others do not. In interpreting the available results, however, caution is required. There are differences between published studies with regard to sample size, LC administration route, dose, and duration of therapy. Published studies may also exclude clinical trials which were not completed because of negative findings. Moreover, some studies have shown the efficacy of LC administration in improving the anemic condition only

in a sub-group of HD patients, retrospectively identified according either to the higher increase of LC plasma levels during active treatment (Caruso *et al.*, 1998) or higher baseline plasma and RBC LC level (Kletzmayr *et al.*, 1999). The apparent dichotomy between responders and non-responders to LC treatment may depend on the proportion of non-acetyl acyl-carnitines within the total carnitine pool (Reuter *et al.*, 2009).

Even though small case series have been published, according to the latest clinical practice guidelines for evaluation and management of anemia in CKD (KDIGO Anemia Work Group, 2012), there is not sufficient evidence upon which to base a recommendation for using LC as an adjuvant to ESA treatment. However, LC use has been suggested in dialysis patients with documented LC deficiency in whom all other conventional measures to raise Hb have been attempted and failed (Yee, 2012).

As discussed above, the most common therapeutic use of carnitine regards the parenteral treatment of its deficiency occurring in ESRD patients in HD. However, whether this kind of secondary deficiency may result in a pathologic phenotype requiring carnitine supplementation has been a matter of controversy (Schreiber, 2005; Steinman, 2005). While there is no doubt that severe forms of carnitine deficiency, such as the very rare one caused by a defect in the carnitine transporter (Tein, 2003), may not be compatible with life if left untreated, doubts have been raised as to the use of carnitine to correct the more modest deficiency often seen in HD patients (Steinman, 2005; Wasserstein, 2013). The partial success of carnitine therapy in improving uremic anemia has even been ascribed to the presence of HD patients responding to carnitine therapy (Kletzmayr *et al.*, 1999; Matsumoto *et al.*, 2001; Reuter *et al.*, 2009), whereas it is not yet clear what makes these patients respond with a favorable clinical outcome. One possible explanation for such partial response to carnitine therapy may be carnitine exposure and dosage. Commonly, carnitine is administered IV at the end of each dialysis session at a dosage of 10-20 mg/kg, though higher and lower dosages have also been used. In this context, it has been shown that at 10 mg/kg plasma levels of free carnitine have already reached values three to four times above the physiological concentration and that, as expected, by increasing the dose to 40 mg/kg plasma carnitine exposure was tenfold higher than normal plasma levels (Brass *et al.*, 2001). Therefore, one may assume that in the majority of clinical studies, where carnitine has been administered at the end of each dialytic session at a dose of 5 mg/kg and above, carnitine deficiency was not only fully corrected, but a supra-physiological exposure was achieved. Thus, a legitimate question arises: if carnitine treatment results in a favorable clinical outcome in HD patients (improvement of uremic anemia), does this originate from the correction of a carnitine deficiency status (as frequently discussed in the literature) or does it mirror a pharmacological effect due to the achievement of supra-physiological carnitine levels. If the latter were the case, what would be the optimal dosage/exposure required to fully exploit the potential anti-anemic actions of carnitine in HD patients? Again, several studies have shown that carnitine exerts a pharmacological action both *in vitro* and *in vivo* at concentrations higher than those normally present in the extra- and intra-cellular milieu (Arduini *et al.*, 2008). It is time to change the paradigm and to start considering carnitine as a 'conditional drug' rather than as a 'conditional vitamin'.

15.4 Conclusions

Despite long-term clinical use and a large number of published trials, the therapeutic role of LC in the anemia of ESRD is still debated. However, the appropriate concentration/exposure that needs to be achieved to fully exploit the beneficial effects of LC remains to be determined. Indeed, the concept of supra-physiological LC therapy has not been investigated in the ESRD population. This issue should be addressed in future studies, considering the experimental evidence discussed above and the excellent safety profile of LC even at high plasma exposure (Brass *et al.*, 2001), hopefully helping to resolve the debate as to the role of LC in anemic ESRD patients on dialysis.

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Foods, vitamins and minerals in leukemia

16. Oxidative stress, leukemia growth and possible therapies

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Abstract

Oxidative stress or reactive oxygen species (ROS) generation is known to mediate a network of intracellular signaling that may cause deleterious effects including cell damage and abnormal physiological functions. Oxidative stress normally refers to altered redox status such as imbalance of oxidized glutathione or nicotinamide adenine dinucleotide phosphate (NADPH/NADP+) ratios. Escalated ROS generation can cause oxidative stress, which has been considered a causative factor of various diseases including cancer. Oxidative stress has been known to promote hematopoietic malignancies including acute and chronic leukemogenesis. Leukemia represents a large heterogeneous group of diseases, such as acute and chronic myeloid leukemia and acute and chronic lymphoblastic and lymphocytic leukemia. In leukemogenesis, acute myeloid leukemia is characterized by highly proliferative hematopoietic blast cells, which undergo abnormal differentiation and proliferation. This abnormal differentiation and proliferation of blast cells is associated with hyper leukocytosis, extra medullary disease, and abnormal coagulation. The underlying molecular mechanism regarding initiation, development and progression of leukemogenesis by oxidative stress needs to be investigated. However, a deep study of ROS generation in leukemia can open new opportunities for effective pharmacological interventions including chemo-radio and immune therapies as well as targeted therapies for del(17p) or TP53. These various possible therapies target survival pathways such as Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT signaling and ultimately inhibit leukemia progression and growth. This chapter reviews the recent knowledge regarding the potential role of oxidative stress in the pathogenesis of leukemia and further outlines possible therapies targeting oxidative stress in leukemia malignancies.

Keywords: reactive oxygen species, signaling, treatment

Key facts

- Leukemia will be the sixth leading cause of death in the USA in 2015.
- Leukemia particularly affects older people and younger children.
- The underlying molecular mechanism of leukemia development remains to be investigated.
- Excessive reactive oxygen species generation contribute to leukemia development.
- Currently available chemotherapeutics need to be tailored for better ef cacy.

Summary points

- Oxidative stress regulates cell proliferation and growth as well as control cells survival.
- Oxidative stress causing cell damage and abnormal physiological functions.
- The underlying mechanism regarding the malignant role of oxidative stress remains elusive.
- Excessive reactive oxygen species, causing malignant transformation and poor prognosis in leukemia.
- Oxidative stress induces DNA mutations by causing double-strands break, genome instability, and enhances leukemia cells proliferation.
- Oxidative stress regulates survival, PI3K and Nrf2 pathways in leukemia.
- Targeted therapies can prevent oxidative stress-induced leukemia development.

Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CML	Chronic myeloid leukemia
COX-2	Cyclooxygenase-2
ERK	Extracellular signal-regulated kinase
FLT3	FMS-like tyrosine kinase-3
GPx	Glutathione peroxidase
GSH	Glutathione
HSCT	Hematopoietic stem cell transplantation
IL	Interleukin
ITD	Internal tandem duplication
MEK	MAPK/ERK kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
PDKs	Phosphoinositide-dependent kinases
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription

16.1 Introduction

ROS are heterogeneous chemically active signaling molecules that play an important role in modulating biological cellular processes, signal transduction pathways, and homeostasis (D'Autréaux and Toledano, 2007; Finkel, 2003; Zhang *et al.*, 2011). Importantly, ROS are derived from superoxide (O_2^-), generated by the mitochondria or nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and have been reported to mediate cell-cycle transition, cell growth by regulating redox-sensitive transcriptional factors, enzymes, oncogenes in a variety of cell types (Jitschin *et al.*, 2014). In contrast, studies have shown that optimum or low levels of ROS exert beneficial effects, regulating cell survival pathways and ultimately promote cell proliferation and growth. Frequently, an adequate balance between producing and eliminating ROS is maintained in cells by an antioxidant defense mechanism or specific enzymatic substrate interaction that control over-production of ROS. Studies have shown that over-production of ROS is associated with oxidative stress, causing DNA damage and modulation of regulatory proteins that support cancer progression and development (Toyokuni, 2006).

There is compelling evidence that oxidative stress contributes to abnormal proliferation and growth of leukemia cells, but the underlying mechanism regarding the malignant role of oxidative stress remain elusive (Finkel, 2003; Kumar *et al.*, 2008). Studies have shown that cancer cells as well as activated granulocytes have the ability to generate excessive ROS, causing malignant transformation and poor prognosis (Kamiguti *et al.*, 2005; Yamaura *et al.*, 2009). Excessive ROS generation modulates the expression of redox-sensitive regulatory proteins, causing genetic

instability and drug resistance in leukemia including ALL and CLL, MDS and myeloid leukemia including AML and CML (D'Autréaux and Toledano, 2007; Trachootham *et al.*, 2009). According to the American Cancer Society, it is estimated that a total of about 52,380 (30,100 men and 22,280 women) new cases will be diagnosed, while 24,090 patients (14,040 men and 10,050 women) are expected to die from leukemia in 2014 (Siegel *et al.*, 2015). Among leukemia, a total of 6,020 new cases and 1,440 deaths due to ALL; 15,720 new cases and 4,600 deaths due to CLL; 18,860 new cases and 10,460 deaths due to AML; 5,980 new cases and 810 deaths due to CML; and 5,800 new cases and 6,780 deaths due to other leukemia are expected to occur in the USA in 2014 (Siegel *et al.*, 2015).

Current therapeutic strategies appear to have reached the limit of their effectiveness; thus, novel strategies are urgently needed to target leukemic cells exclusively. Several recent studies have demonstrated that altered antioxidant status is a distinct feature of AML, and in blasts isolated from patients with various types of leukemia, oxygen radical levels were significantly higher in malignant cells than in normal leucocytes (Er *et al.*, 2007; Li *et al.*, 2011; Zhou *et al.*, 2010). Altered intracellular and extracellular ROS trigger acute myeloid leukemogenesis. Malignant cells on their part appear adapted to permanent (intrinsic) oxidative stress and up-regulate protective pathways, which in addition favor resistance toward anticancer agents (Wu *et al.*, 2010). Increasing evidence suggests a connection between tumor specific metabolism and a surplus of ROS production. Metabolic alterations can render malignant cells more susceptible to perturbations within the metabolic framework and therefore pose opportunity for novel therapeutic interventions (Cheong *et al.*, 2012).

In this chapter, we review the recent medical literature and address the role of intracellular and extracellular ROS in acute myeloid leukemogenesis. Opportunities for pharmacological intervention are identified to demonstrate how recent findings may facilitate the implementation of redox therapies for AML. Extensive research during the past two decades has revealed the mechanism by which continued oxidative stress can lead to chronic inflammation, which in turn could mediate most chronic diseases including cancer, diabetes, and cardiovascular, neurological, and pulmonary diseases.

16.2 Altered oxidative stress promotes leukemogenesis

Oxidative stress or imbalance in production of free radicals, results in detrimental effects in the cellular environment as well as adversely regulate the signaling mediated gene expression (Durackova, 2009). Mostly, ROS are generated in cells by the mitochondrial respiratory chain. Studies have shown that altered oxidative stress induces leukemia cell growth and consequently development of leukemia (Goossens *et al.*, 1995, 1999). Under continuous oxidative stress, ROS are produced for a significant period of time and damage cell structure and functions. This results in somatic mutations and neoplastic transformations of hematopoietic cells (Fang *et al.*, 2009; Khandrika *et al.*, 2009).

16.3 Oxidative stress-induced genetic instability in leukemia

Cancer initiation and development has been linked to oxidative stress because oxidative stress induces DNA mutations by causing double strands break, genome instability, and enhances leukemia cells proliferation (Visconti and Greico, 2009). Inherited and sporadic human leukemias can be accredited to genetic instability as a result of DNA damage and failed DNA repair (Popp and Bohlander, 2010). Increased levels of ROS can lead to constitutive activation of transcription factors and their respective genes, which results in the activation of the genes involved in the malignant phenotype. This is one of the many ways in which oxidative stress plays a role in the progression of cancer and the development of the many stages of carcinogenesis (Toyokuni *et al.*, 1995).

Recent studies have linked oxidative stress to malignant diseases such as childhood ALL. Oxidative stress is expressed in the form of lipid peroxidation products or other plasma states (lmbesi *et al.*, 2013). High levels of oxidation modified DNA bases in the lymphocytes of children with ALL, together with decreased levels of antioxidant enzymes (Almondes *et al.*, 2014).

Many oncogenes are capable of altering the redox balance of leukemic cells. These include KRAS, cMYC, BCR/ABL, NRF2, and NF-κB (Kindler *et al.*, 2010; Kiyoi *et al.*, 1999). The fusion gene, BCR/ABL, found in CML is shown to be modulated with ROS levels in both human and murine cell lines (Kim *et al.*, 2005; Sattler *et al.*, 2000). BCR/ABL-induced ROS also disrupts signaling that leads to the up-regulation of the non-receptor tyrosine kinase FYN (Ban *et al.*, 2008; Gao *et al.*, 2009). Low levels of FYN in the presence of the active BCR/ABL gene act as a mediator for the development of CML and are also responsible for the drug-resistance of CML patients particularly to the BCR/ABL inhibitor, imatinib. Additionally, mutations of ITD of the FLT3 receptor have been found in approximately 25% of new cases of AML (Kindler *et al.*, 2010). FLT3-ITD has been found to induce high levels of ROS within murine Ba/F3 or 32D cells as well as MOLM-14 and MV-4-11 human AML cell lines, which carry FLT3-ITD mutations (Sallmyr *et al.*, 2008). This suggests the importance of ROS in regulating FLT3 mutated AML.

16.4 Inflammation contributes to leukemia growth via reactive oxygen species

Inflammation is an adaptive physiological response induced by deleterious circumstances including infection and tissue injury. Observational studies have revealed that inflammation is the product of a complex series of responses triggered by the immune system. Inflammation also harbors a wide range of physiological and pathological morbidities (Ruslan, 2008). Extensive research has shown that inflammation is associated with alteration of signaling pathways, which results in increased levels of inflammatory markers, lipid peroxides, and free radicals. It has also been hypothesized that inflammation plays a central role in the wound healing and combating infection. However, persistent inflammation can activate the immune system for long durations, which stimulates the progression of chronic diseases such as pulmonary, cardiovascular, metabolic, and neurologic diseases, as well as cancers (Dantzer *et al.*, 2008; Ruslan, 2008).

During inflammation, mast cells and leucocytes are recruited to the site of damage, which leads to a 'respiratory burst' due to an increased uptake of oxygen, and thus, increased release and accumulation of ROS at the site of damage (Coussens and Werb, 2002; Hussain and Harris, 2003). On the other hand, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines and chemokines, which recruit inflammatory cells to the site of damage and producing more reactive species. These key mediators can activate signal transduction cascades as well as induce changes in transcription factors, such as NF-κB, STAT3, hypoxia-inducible factor-1α (HIF1-α), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NF-E2 related factor-2 (Nrf2), which mediate immediate cellular stress responses. Induction of COX-2, inducible nitric oxide synthase (iNOS), aberrant expression of inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1, IL-6 and chemokines (IL-8 and CXC), chemokine receptor 4 (CXCR4), as well as alterations in the expression of specific microRNAs, have also been reported to play a role in oxidative stress-induced inflammation (Hussain and Harris, 2007). This sustained inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighboring epithelial and stromal cells and over a long period of time may lead to carcinogenesis (Federico *et al.*, 2007).

COX-2 has been implicated in the development of many hematological cancers, as well as in tumor angiogenesis. COX-2 inhibitors have been shown to have anti-tumor activity in experimental cancer model. In a recent study, expression of COX-2 has been detected in both lymphocytes (T-cells and B-cells) whereas little or no expression was observed in unstimulated cell-lines. In AML cell lines, COX-2 expression was almost universal and easily detectable. Furthermore, survival and growth of AML cell lines was inhibited by COX-2 inhibitors celecoxib and NS398. COX-2 expression increases with aging in most tissues, due in part to ROS, chemical reactions, physical shearing, and dietary molecules (Luo *et al.*, 2011).

16.5 Oxidative stress-regulated cell signaling in leukemia

There are three main pathways that are frequently associated with the oxidative stress-induced leukemia. The Ras, Raf, MEK-1/-2 (mitogen-activated protein kinase/ERK kinase) pathway, p13k/PTEN/Akt/mtor pathway and the Jak/STAT pathway.

16.5.1 Ras/Raf/ERK pathway

In the ERK signaling pathway, ERK (ERK1 and ERK2) is activated when phosphorylated by MEK (MEK1 and MEK2), which is activated when phosphorylated by Raf (Raf-1, B-Raf and A-Raf) (Morrison and Davis, 2003). Most of the cancer-associated aberrations that cause constitutive activation of ERK signaling occur at the early stages of the pathway such as over expression of receptor tyrosine kinases, activating mutations in receptor tyrosine kinases, Ras mutations and B-Raf mutations. ERK signaling is capable of driving a diverse array of cellular processes. Depending on the particular cell type these processes include proliferation, differentiation, survival, migration, angiogenesis and chromatin remodeling (Dunn *et al.*, 2005; Yoon and

Seger, 2006). It has been reported that more than 50% cases of AML and ALL have constitutively activated Raf/MEK/ERK pathway without the presence of any known genetic mutation (Ricciardi *et al.*, 2005; Kornblau *et al.*, 2006). As mentioned before the FLT-3 kinase, which is the FMS like tyrosine kinase, is mutated in more than 25% of AML cases. Similarly the BCR/ABL mutation which is present in more than 95% of CML patients and some ALL patients, can also lead to the abnormal activation of this signaling cascade (Steelman *et al.*, 2008).

When breast cancer patients were treated with chemotherapy, they developed therapy-induced acute mylogenous leukemia (t-AML). Mutated forms of the RAF1 and the BRAF genes were detected in these leukemias (Christiansen *et al.*, 2005; Zebisch *et al.*, 2006). Further studies revealed that the mutated RAF-1 gene was transmitted through the germ line and was therefore thought to be responsible for the induction of t-AML in these patients. Many mutated genes that code for receptor tyrosine kinases involved in the Ras/RAF cascade have been detected in therapy related AML and myelodysplasia (Christiansen *et al.*, 2005).

16.5.2 JAK/STAT pathway

STAT proteins are a family of cytoplasmic transcription factors, which are activated by growth factors, cytokines and hormones and participate in diverse processes such as proliferation, differentiation and survival (Darnell, 1997; Imada and Loenard, 2000; Takeda and Akira, 2000; Williams, 2000). Cytokines or growth factors bind to their cognate receptors, which leads to receptor oligomerization. This activates a kinase signaling cascade in the receptor cytoplasmic domain (Heldin, 1995). Activation is also achieved by receptor-associated tyrosine kinases including the JAKs (Rane and Reddy, 2002) or Src family kinases (SFKs) (Chaturvedi *et al.*, 1998). Activation of this kinase signaling leads to direct phosphorylation of tyrosine residues of STAT proteins followed by STAT dimerization. The homo- or hetero-dimerized STAT proteins then translocate to the nucleus where they bind to specific promoter regions and induce gene transcription.

Evidence suggests that STAT3 contributes to cell cycle arrest by regulating the transcription of the cyclin dependent kinase inhibitor $p27^{Kip1}$ (Koning *et al.*, 2000). It may also advance differentiation by up-regulating the expression and transcriptional activity of CCAAT/enhancer binding protein α (C/EBPa), a key transcription factor involved in myeloid differentiation (Numata *et al.*, 2005).

Similarly, STAT5 can also regulate the activity of the anti-apoptotic proteins such as Bcl2 and Bcl-Xl by enhancing its transcription and therefore leading to the survival and progression of myeloid progenitors (Kieslinger *et al.*, 2000). STAT1, unlike STAT3 and STAT5, is induced and activated by all-trans retinoic acid (ATRA) during myeloid differentiation of various leukemia cell lines (Gianni *et al.*,1997).

16.5.3 PI3K/PTEN/Akt/mTOR

The PI3K/AKT and mTORC1 pathways can regulate both normal and malignant hematopoiesis (Yilmaz *et al.*, 2006; Zhang *et al.*, 2006). PI3K activity controls erythropoiesis by mediating the proliferation and survival of erythroid progenitors (Bouscary *et al.*, 2003; Myklebust *et al.*, 2002). AKT on the other hand regulates lineage fates during myelopoiesis which involves the control of phosphorylation of the transcription factor CEBPA. When AKT is inactivated, GSK-3 activation takes place, which results in phosphorylation and inactivation of CEBPA, and in turn causes eosinophil differentiation, whereas AKT activation activates CEBPA and enhances neutrophil development (Buitenhuis *et al.*, 2008). Therefore, an aberrant PI3K/AKT pathway may play a key role in the progression of leukemia.

Activated PI3Ks produce phosphoinositide-3-phosphate and phosphoinositide-3,4,5-triphosphate which are required for the activation of PDK1 and PDK2 (Vanhaesebroeck and Alessi, 2000). The PDKs, in turn, activate Akt, also known as protein kinase B, by phosphorylating Akt on key serine and threonine residues (Nicolson and Anderson, 2002). The known biological functions of Akt include the regulation of glucose transport and promotion of cell survival. Akt exerts its anti-apoptotic effects via phosphorylation of proteins involved in apoptosis regulation (Datta *et al.*, 1997). In an experiment, human leukemia cells (HL-60) were cloned with a constitutively active PI3K/Akt gene. It was seen that these cells were resistant to As_2O_3 induced apoptosis, while normal parental cells were sensitive to apoptosis (Tabellini *et al.*, 2005a,b). Through this it can be deduced that the PI3K/Akt pathway protects cells from apoptosis induced by ROS and an inhibitor of this pathway and ROS production through As_2O_3 could be used together to induce apoptosis in leukemic cells. This was confirmed by experiments where siRNA knockdown of Akt promotes apoptosis induced by As_2O_3 in leukemic cell lines such as HL-60 and MOLT-4. Apoptosis is induced by As_2O_3 by inhibition of GPx. GPx is involved in the reduction of H_2O_2 and lipid hydroperoxides (Tabellini *et al.*, 2005a; 2005b).

16.5.4 NRF2 pathway

It has been reported that constitutively activated NRF2 has been detected in malignant blasts isolated from AML patients, that lead to increased cell survival and resistance to chemotherapy (Rushworth *et al.*, 2011, 2012). The same has been seen in patients with CLL (Hayes and McMahon, 2009). Furthermore, constitutive activation of NRF2 has been linked to the activation of NF-κB. The link between NRF2 and GSH is such that the key enzymes involved in the regulation of GSH synthesis, are in turn regulated by NRF2 through activation of ARE (Xue *et al.*, 2013). AML cells have been reported to have increased levels of enzymes that metabolize GSH, such as GCL and GST compared to normal CD34+ cells (Pei *et al.*, 2013). Furthermore a decrease in the ratio of GSH to GSSG (oxidized form of GSH regulated by GPx) and increased levels of NRF2 go to show that leukemic cells compensate for the increased oxidative stress through aberrant activation of the NRF2 pathway (Rushworth *et al.*, 2011). The link between GSH and NRF2 can serve as an important mediator in leukemia therapy because GSH has also been linked with resistance to chemotherapy.

16.6 Possible therapies for leukemia

Leukemia therapy has been significantly enhanced over the past 20 years by investigating the effect of oncogenes-regulated cellular proliferation and cell membrane-permeable inhibitors that suppress the proteins critically required for the growth of the leukemic cells. Traditional treatment strategies for the prevention of leukemia include stand-alone chemotherapy, or chemotherapy in combination with radiation therapy or stem cell transplantation (Fausel, 2007; Gokbuget *et al.*, 2006; Hegenbart *et al.*, 2006). The standard chemotherapy regimen either kills malignant cells to induce remission, or damage them to inhibit the progression of disease. In general, combinations of drugs are used to induce early remission and a lower risk of disease resistance. Consolidation and maintenance treatments are intended to prevent disease recurrence. Consolidation treatment often entails a repetition of induction chemotherapy or the intensification of chemotherapy with additional drugs. By contrast, maintenance treatment involves drug doses that are lower than those administered during the induction phase (Gokbuget *et al.*, 2006). Meanwhile, high dose chemotherapy may be used to treat refractory or relapsed leukemia which is then followed by bone marrow rescue in the form of autologous or allogeneic HSCT (Ishaqi *et al.*, 2008). Graft-versus-leukemia effects are induced by allogeneic HSCT and this is an important mechanism of leukemia suppression (Copelan, 2006). In cases of post-HSCT relapse, donor lymphocyte infusion can be effective (Huang *et al.*, 2007). However, allogeneic transplantation is limited by suitable donor availability and by transplant-associated mortality and morbidity (Devetten and Armitage, 2007; Petzer and Gunsilius, 2003). Targeted therapy has increased the effectiveness of chemo-, radio- and hormonal-based therapies (McCubrey *et al.*, 2008).

16.6.1 Targeting the Raf/MEK/ERK pathway

An advantage of inhibiting the Raf/MEK/ERK cascade is that it can be targeted without knowledge of the precise genetic mutation, which results in its aberrant activation. This is important as the nature of the critical mutation(s), that leads to the malignant growth of at least 50% of AMLs and other cancers, is not currently known. An advantage of targeting MEK is that the Raf/MEK/ERK pathway is a convergence point where a number of upstream signaling pathways can be blocked with the inhibition of a single kinase (MEK). Some of the MEK inhibitors are being evaluated in combination with inhibitors which target other signaling and anti-apoptotic molecules to improve leukemia therapy (Milella, 2005).

Raf inhibitors have been developed and some (sorafenib) are being considered for evaluation in leukemia trials. Certain Raf inhibitors have been developed which are small molecule competitive inhibitors of the ATP-binding site of Raf protein. These inhibitors (for example, L-779,450, ZM 336372, Bay 43-9006, also known as sorafenib) bind the Raf kinase domain (KD) and therefore prevent its activity (Hall-Jackson *et al.*, 1999; Lyons *et al.*, 2001; Shelton *et al.*, 2003; Zhang *et al.*, 2008). Some Raf inhibitors may affect a single Raf isoform (for example, Raf-1), others may affect multiple Raf proteins, which are more similar (Raf-1 and A-Raf), and while other pan Raf inhibitors may affect all three Raf proteins (Raf-1, A-Raf and B-Raf), sorafenib inhibits other kinases besides Raf (for example, VEGF-II receptor, PDGF-R, Kit, Flt-3, Fms) and is more

appropriately referred to as a multi-kinase inhibitor. Knowledge of the particular RAF gene mutated or overexpressed in certain leukemia may provide critical information regarding how to treat the leukemia patient; some patients who overexpress a particular RAF gene may be more sensitive to inhibition by agents which target that particular Raf protein. Thus the development of unique and broad-spectrum Raf inhibitors may prove useful in leukemia therapy.

Chaperonin proteins such as 14-3-3 and Hsp90 regulate Raf activity (Blagosklonny, 2002; Workman, 2004). Raf activity is regulated by dimerization. These biochemical properties result in Raf activity being sensitive to drugs which block protein-protein interactions such as geldanamycin (Blagosklonny, 2002; Workman, 2004). Modified geldanamycins may be useful in treatment of CMLs which are resistant to BCR-ABL inhibitors. We often think of a single Raf protein carrying out its biochemical activity. However, Raf isoforms dimerize with themselves and other Raf isoforms to become active. Drugs such as coumermycin, which inhibit Raf dimerization and others such as geldanamycin, which prevent interaction of Raf with Hsp90 and 14-3-3 proteins suppress Raf activity.

An alternative approach to targeting Raf is to prevent Raf activation by targeting kinases (for example, Src, protein kinase C (PKC), PKA, p21-activated protein kinase (PAK) or Akt) and phosphatases (for example, protein phosphatase-2A (PP2A)) involved in Raf activation. Some Src kinase inhibitors such as dasatinib would be predicted to inhibit Raf activation as it should suppress Raf-1 activation by Src (Mccubrey *et al.*, 2008)

Aberrant activation of MEK1 is observed in many different leukemias due to the activation of the Raf/MEK/ERK pathway by upstream kinases (for example, BCR-ABL) and growth factor receptors (for example, PDGF-R, Kit, Flt-3, Fms) as well as other unknown mechanisms. Specific inhibitors to MEK have been developed (PD98059, U0126, PD184352 (also known as CI1040), PD-0325901, Array MEK inhibitors (ARRY-142886 and others) (Milella *et al.*, 2005).

Abnormal activation of ERKs, and their upstream activators, has been reported in AML (Bardet *et al.*, 2006). A specific inhibitor of MEK, AZD6244, which also blocks signaling via ERK1/2, was shown to effectively inhibit proliferation of leukemic cells (Nishioka *et al.*, 2008). Statins were able to down-regulate constitutive ERK activation in AML cell lines as well as in AML primary blasts which could contribute to apoptosis (Wu *et al.*, 2004). Finally, farnesyl-transferase inhibitors, which target a key post-translational modification of the RasGTPase, have been shown to be effective against various cancers including leukemia (Wojtkowiak *et al.*, 2009).

16.6.2 Targeting thePI3K/PTEN/Akt/mTOR pathway

The PI3K/PTEN/Akt/mTOR pathway may be inhibited with several small molecules. For PI3K inhibition (LY294002, PX-866), PDK1 (OSU-03012, celecoxib), for Akt inhibition (A-443654, perifosine, tricribine) or downstream mTOR inhibitors such as rapamycin and modified rapamycins (CCI-779 and RAD001) has been practiced (Martelli *et al.*, 2006, 2009; Papa *et al.*, 2008). PI3K is a lipid kinase that is regulated in response to multiple hematopoietic cytokines

and chemokines, including the Flt3 receptor (Grandage *et al.*, 2005; Xu *et al.*, 2003). Many of the downstream effects of PI3K are mediated through the serine-threonine protein kinase Akt and the downstream mammalian target of rapamycin (mTOR) (Xu *et al.*, 2003), with constitutive activation in AML blast cells, strongly influencing leukemic cell proliferation, survival and chemo-resistance. Blocking of PI3-K rapidly induces apoptosis, suggesting that inhibitors of this pathway representing attractive pharmacological agents for the treatment and prevention of leukemia (Levy *et al.*, 2008; Plate, 2008; Prevo *et al.*, 2008).

16.6.3 Targeting the JAK/STAT pathway

STAT proteins are highly expressed in leukemias and lymphomas regulating diverse processes such as apoptosis, signaling and angiogenesis (Bowman *et al.*, 2000). Different isoforms of STAT have been reported, that are overexpressed in leukemia. STAT1 is activated in AML and ALL (Bowman *et al.*, 2000), STAT3 is expressed in AML (Benekli *et al.*, 2002; 2003), whereas STAT5 is activated in ALL, AML, APL, CML, AMKL, and MPDs (Arnould *et al.*, 1999; Lewis and Ward, 2008; Sternberg and Gilliland, 2004; Ward *et al.*, 2000). STAT activation is also observed in genetic transformations by oncoproteins such as BCR-ABL (Shuai *et al.*,1996), TEL-JAK2 (Lacronique *et al.*,1997), STAT5b-RAR (Dong and Tweardy, 2002) and FLT3-ITD (Choudhary *et al.*, 2007). HTLV-I-infected T cells also express active STATS wherein they are resistant to apoptosis (Migone *et al.*, 1995; Silver *et al.*, 2004; Tomita *et al.*, 2006).

A number of strategies are in the pipeline aiming to specifically target STAT proteins (Turkson, 2004). These strategies controlled the expression of STAT proteins by reducing their expression level; e.g. STAT3 gene is silenced by antisense oligo deoxyribonucleic acids, which reduces proliferation of leukemias and lymphomas *in vitro* (Jing and Tweardy, 2005). STAT5 is activated by the oncogene BCR-ABL, which can be silenced through RNAi (Scherr *et al.*, 2006). Peptide or oligonucleotide decoys are also being used as alternative means for inhibition of STAT proteins (Jing and Tweardy, 2005; Turkson *et al.*, 2001)

16.6.4 JAK2 inhibitors

Considering the role of JAK2 in various multiple proliferative disorders, several inhibitors targeting JAK-2 are being developed (Pardanani, 2008). An example of such an inhibitor is AG490, which inhibits cell proliferation both *in vivo* and *in vitro* in leukemia, without effecting normal hematopoiesis. AG490 is a protein kinase inhibitor and belongs to the tryphostin family (Meydan *et al.*, 1996; Miyamoto *et al.*, 2001). WP1066 is an inhibitor that can target isoforms of JAK2 such as the JAK2 V617F in erythroid leukemia by blocking cell proliferation (Verstovsek *et al.*, 2008). Erlotinib is an EGFR inhibitor and it has been shown to be effective in suppressing the proliferation of hematopoietic progenitor cells having JAK2 V617F mutation (Li *et al.*, 2007). These drugs are undergoing phase I studies and are therefore restricted to patients with a high risk of MPD (Mullighan *et al.*, 2009; Pardanani, 2008).

16.7 Conclusions

ROS modulate the expression of redox-sensitive transcription factors, enzymes, oncogenes, and other cell signaling effectors (Lyu *et al.*, 2008). Recent advances in our understanding of AML biology have contributed to better identification of genetic and epigenetic abnormalities in leukemic cells and ROS-mediated leukemic hematopoiesis. Abnormal ROS levels are characteristic of different types of leukemia and central target for the development of anti-leukemic drugs (Grek *et al.*, 2011). Thus, targeting ROS as an antileukemic strategy is expected to provide a favorable clinical benefit for leukemia patients.

Targeting the frequently activated pathways like the Raf/MEK/ERK pathway or the PI3K/PTEN pathway has been the focus of research. Many drugs have been developed that inhibit important mediators involved in the progression of these pathways. According to recent evidence the BRAF gene mutation in cancers lead to cell proliferation (Flotho *et al.*, 1999) whereas only a few years ago the mutated BRAF-1 gene was thought to promote cell cycle arrest (McCubrey *et al.*, 2007). An intriguing aspect of leukemia therapy is that in some cases stimulation of the Raf/MEK/ERK pathway may be desired to promote terminal differentiation, while in other types of malignant cancers inhibition of the Raf/MEK/ERK pathway may be suppress proliferation. Similarly, the phosphorylation events mediated by Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways are associated with the prevention of apoptosis. Thus, a deep understanding of these molecular pathways may provide a plate form for efficient agents or drugs that could potentially inhibit the development and progression of leukemia through modulation of oxidative stress.

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17. Genetic variants of folate metabolic pathways in hematological toxicity of leukemia patients

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Abstract

Two chemotherapeutic agents, 6-mercaptopurine and methotrexate, which are part of consolidation therapy in leukemia, are found to be associated with hematological toxicity in some patients. S-adenosyl methionine, the metabolic byproduct of folate pathway, is shown to stabilize thiopurine methyl transferase activity and controls the toxic levels of the 6-thioguanine nucleotide by methylating most of the metabolic products of 6-mercaptopurine. Existing literature points to the association of the genetic variants of the folate pathway, such as glutamate carboxypeptidase II C1561T, reduced folate carrier 1 G80A and thymidylate synthase (TYMS) 5'-UTR 28bp tandem repeat, with hematological toxicity either alone or in conjunction with thiopurine methyl transferase variant alleles. Methotrexate, which inhibits two crucial enzymes of folate pathway i.e. dihydrofolate reductase and TYMS, is also found to induce toxicity in the presence of reduced folate carrier 1, G80A, TYMS 5'-UTR 28bp tandem repeat, methylene-tetrahydrofolate reductase (MTHFR) C677T, methionine synthase reductase A66G polymorphisms. Furthermore, survivors of childhood ALL are found to develop attention deficit hyperactivity disorder, when they harbor MTHFR C677T, MTHFR A1298C and methionine synthase A2756G polymorphisms. These cognitive defects are attributed to low levels of 5-methyl tetrahydrofolate and S-adenosyl methionine in the cerebrospinal fluids of such children following chemotherapy. Gene expression analysis reveals differential expression of certain folate pathway genes in subtypes of acute lymphoblastic leukaemia, based on lineage (T-cell or B-cell), hyperploidy or non-hyperploidy and presence or absence of gene fusion, suggesting that methotrexate dynamics might vary, depending on subtypes of acute lymphoblastic leukemia. To conclude, folate pathway gene expression and genetic analysis might help in predicting patients at risk of developing all types of toxicity, including hematological toxicity in leukemia.

Keywords: mercaptopurine, methotrexate, glutamate carboxypeptidase II, reduced folate carrier 1, thymidylate synthase

Key facts

- S-adenosyl methionine stabilizes thiopurine methyl transferase activity and reduces toxicity associated with 6-mercaptopurine and its metabolites.
- Folate pathway genetic polymorphisms such as glutamate carboxypeptidase II (GCPII) C1561T, reduced folate carrier 1 (RFC1) G80A and thymidylate synthase (TYMS) 5′-UTR 28bp tandem repeat are associated with 6-mercaptopurine mediated hematological toxicity either alone or in conjunction with thiopurine methyl transferase (TPMT) variant alleles.
- RFC1 G80A, TYMS 5′-UTR 28bp tandem repeat, methylenetetrahydrofolate reductase (MTHFR) C677T and methionine synthase reductase (MTRR) A66G polymorphisms are associated with methotrexate-mediated toxicity.
- The survivors of childhood acute lymphoblastic leukemia (ALL) experience cognitive deficits following chemotherapy when they harbor MTHFR C677T, MTHFR A1298C and methionine synthase A2756G polymorphisms.
- Differential expression of folate pathway genes according to subtypes of ALL influences pharmacodynamics of methotrexate.

Summary points

- The consolidation chemotherapy regimen (6-mercaptopurine and methotrexate) in leukemia induces toxicity in some patients.
- The 6-mercaptopurine mediated toxicity is attributed to genetic variants in TPMT and folate pathway (GCPII C1561T, RFC1 G80A, TYMS 5′-UTR 28bp tandem repeat).
- This association is attributed to stabilization of TPMT activity by S-adenosylmethionine, the final product of folate metabolism.
- The methotrexate-mediated toxicity is attributed to RFC1 G80A, TYMS 5′-UTR 28bp tandem repeat, MTHFR C677T, MTRR A66G polymorphisms, and ALL-subtype specific differential expression of folate pathway genes.
- Perturbations in folate pathway also influence survivors of childhood ALL by inducing cognitive deficits due to low S-adenosylmethionine and low 5-methyltetrahydrofolate.

Abbreviations

6-MP	6-mercaptopurine
ALL	Acute lymphoblastic leukemia
cSHMT	Cytosolic serine hydroxymethyl transferase
DHF	Dihydrofolate
GCPII	Glutamate carboxypeptidase II
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
IMPDH	Inosine-5'-monophosphate dehydrogenase thioxanthine monophosphate
MTHF	Methylene-tetrahydrofolate
MTHFD1	MTHF dehydrogenase
MTHFR	MTHF reductase
MTR	Methionine synthase
MTRR	MTR reductase
MTX	Methotrexate
RFC1	Reduced folate carrier 1
SAM	S-adenosyl methionine
TGMP	Thioguanine monophosphate
TGN	Thioguanine triphosphate nucleotide
THF	Tetrahydrofolate
TIMP	Thioinosine monophosphate
TPMT	Thiopurine methyl transferase
TYMS	Thymidylate synthase
UTR	Untranslated region

17.1 Introduction

Leukemia is the most common type of cancer amongst children. Three quarters of childhood leukemias are ALL. According World Cancer Report by the WHO (Stewart and Wild, 2014), leukemia occurs in 352,000 people worldwide resulting in 265,000 deaths. 6-MP and MTX are part of a poly-chemotherapeutic regimen in leukemia, during the consolidation and maintenance phases of therapy. The pharmacodynamic actions of these two drugs are influenced by folate pathway. This chapter provides a comprehensive overview of pharmacokinetic and pharmacodynamic pathways of these drugs and the influence of genetic alterations in folate pathway on the therapeutic efficacy and toxicity.

17.2 Mercaptopurine metabolism

6-MP is a purine analogue. It is a prodrug. In the body, it is metabolized to TIMP, in the presence of HPRT. IMPDH converts TIMP into TXMP. Guanine monophosphate synthetase catalyzes the conversion of TXMP into TGMP. HPRT catalyzes the conversion of TGMP to 6-TGN. TGN

is incorporated into DNA and interferes with DNA ligases, endonucleases and polymerases. Excess concentration of 6-TGN causes myelosuppression. TPMT activity controls 6-TGN levels by converting 6-MP, TIMP, TGMP and TGN into corresponding methylated derivatives. This conversion can be facilitated only when TPMT is adequate and SAM is available as co-substrate for methylation.

17.3 Folate metabolism

SAM is the metabolic byproduct of folate metabolism. Folate metabolism also known as one-carbon metabolism is characterized by sequential transfer of one-carbon moiety, i.e. methyl or methylene group from one substrate to another to form several crucial substrates. GCPII/folate hydrolase 1 (FOLH-1) expressed in intestinal brush border hydrolyzes dietary folyl polyglutamates into folyl monoglutamates and facilitates intestinal absorption of folate. Folate reductase catalyzes two-step reduction of folate to form DHF and THF. RFC1 helps in bidirectional transport of folate across red blood cell membrane. MTHFD1 is a trifunctional enzyme, which has formate-THF ligase and MTHF cyclohydrolase enzymatic functions, essential for the synthesis of 10-formyl-THF from THF and 5,10-methenyl THF; and also catalyzes the dehydrogenation of 5,10-MTHF to form 5,10-methenyl THF. cSHMT catalyzes transfer of methylene moiety from serine to THF to form 5,10-MTHF. TYMS catalyzes the transfer of methylene moiety from 5,10-MTHF to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). MTHFR catalyzes the thymidylate synthase-mediated reduction of 5,10-MTHF to 5-MTHF. MTR catalyzes the remethylation of homocysteine to methionine using 5-MTHF as a methyl group donor. MTRR supplies the co-enzyme, i.e. methyl cobalamin essential for methionine synthesis, by catalyzing reductive methylation of cobalamin. Methionine combined with ATP to form SAM, the universal methyl donor. SAM donates its methyl group to DNA, proteins and catecholamines and results in the formation of S-adenosyl homocysteine, which gives rise to homocysteine by a hydrolysis reaction.

17.4 Functional relevance of genetic polymorphisms of folate metabolic pathway

Several putatively functional polymorphisms are reported in folate metabolic pathway. GCPII C1561T polymorphism has been reported to impair intestinal absorption of folate (Divyya *et al.*, 2012). RFC1 G80A polymorphism is shown to be associated with reduced circulating folate levels (Naushad *et al.*, 2011). The cSHMT C1420T polymorphism is reported to maintain one-carbon homeostasis by stimulating futile folate cycle. MTHFS metabolizes 5-formyl-tetrahydrofolate to form 5,10-MTHF. The combined activities of MTHFS and SHMT thus maintain one-carbon homeostasis (Stover *et al.*, 1993). Functional analysis of TYMS 5'-UTR 28bp tandem repeat polymorphism revealed reduced TYMS expression with double repeat (2R-allele) in comparison to triple repeat (3R-allele) (Horie *et al.*, 1995). Two polymorphisms in MTHFR, i.e. C677T and A1298C, are found to be distributed worldwide (Schneider *et al.,* 1998). Yamada *et al.* (2001)

developed recombinant human MTHFR using a baculovirus expression system and studied the properties of Ala222Val (C677T) and Glu429Ala (A1298C). The Glu429Ala was indistinguishable from wild-type MTHFR biochemically. The Ala22Val variant exhibited enhanced propensity to dissociate into inactive monomers thus losing its thymidylate synthase-binding capacity. Laraqui *et al.* (2006) observed association of synergistic interaction between MTR A2756G and MTRR A66G polymorphisms with hyperhomocysteinemia. Naushad *et al.* (2008) confirmed this synergistic influence in other ethnic group. Yamada *et al.* (2006) have shown that MTRR maintains MTR activity at a 1:1 stoichiometric ratio by serving as a special chaperone for MTR and as an aquacobalamin reductase. Wolthers *et al.* (2009) showed that reactivation of cob(II) alamin MTR and incorporation of cobalamin into apo-MTR is enhanced through specific protein-protein interactions between the MTRR flavin mononucleotide domain and MTR.

17.5 Methotrexate metabolism

MTX is administered orally or intramuscularly. Oral administration is associated with nausea and intramuscular administration has lesser side effects. RFC1 plays an important role in cellular uptake of MTX. 10% of the absorbed MTX gets hydroxylated to 7-hydroxy MTX in the liver and excreted through renal route and bile. Folylpolyglutamyl synthase converts MTX and 7-hydroxy MTX into polyglutamate derivatives, which are stored in tissues, liver and erythrocytes. The pharmacodynamic effect of MTX is mediated through inhibition of DHF reductase enzyme resulting lower tetrahydrofolate levels, which in turn affect DNA synthesis and DNA methylation. MTX also inhibits 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase and thus contribute to reduced *de novo* purine synthesis (Figure 17.1).

17.6 Evidence for association of gene-gene interactions between thiopurine and folate pathways with toxicity

Deficiency of TPMT or SAM leads to myelotoxicity. Milek *et al.* (2009) observed that SAM reverses the extent of 6-MP cytotoxicity by acting as TPMT-stabilizing factor. Shimasaki *et al.* (2008) observed interruptions in 6-MP and MTX therapy in ALL patients having MTHFR C677T polymorphism, while RFC1 G80A was associated with more frequent interruptions in 6-MP therapy. Karas-Kuzelicki *et al.* (2009) have shown synergistic interactions between TPMT variant alleles and MTHFR genotypes in inflating 6-MP mediated toxicity. Karas-Kuzelicki *et al.* (2010) observed high TPMT activity in men with MTHFR CC-genotype compared to men with CT or TT genotype. Tanaka *et al.* (2014) demonstrated association of MTHFR 677TT and MTHFR 677CT/1298AC with hepatic toxicity during maintenance therapy of childhood ALL (6-MP and MTX). Dorababu *et al.* (2012) reported gene-gene interactions between thiopurine and folate metabolic pathways, i.e. TPMT × 12 × RFC1 G80A; TPMT CTTAT haplotype × RFC1 G80A; TPMT CTTAT haplotype × RFC1 G80A × TYMS 2R3R, inflating 6-MP mediated hematological toxicity. Furthermore, they observed independent association of GCPII C1561T polymorphism with hematological toxicity. The genetic variants of thiopurine and folate metabolic pathways

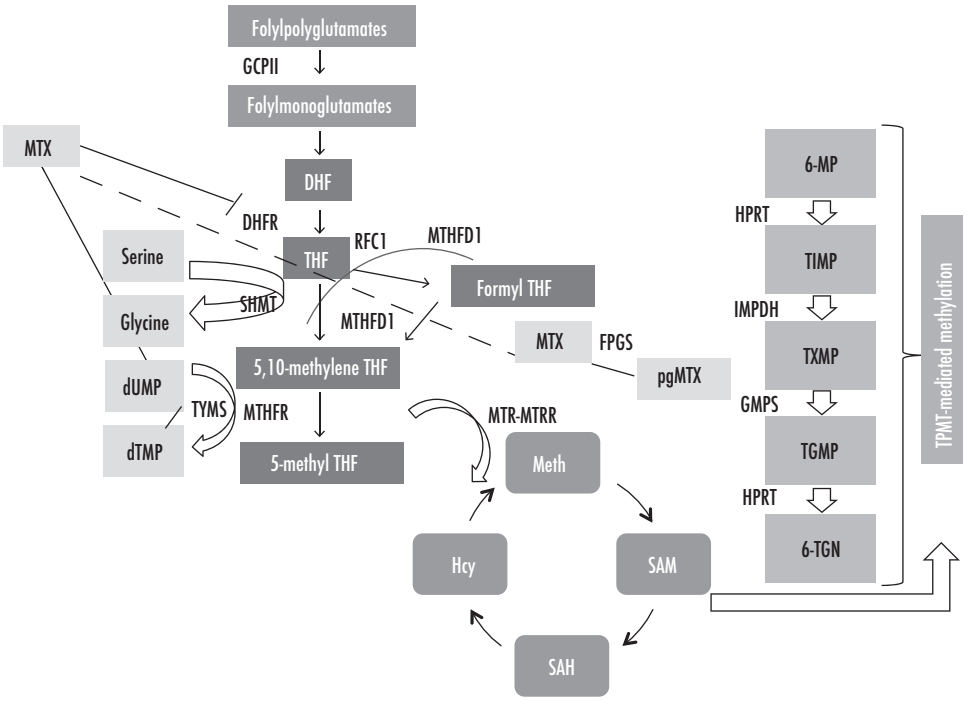

Figure 17.1. Schema depicting the folate metabolic pathway with specific emphasis on 6-mercaptopurine metabolism and methotrexate therapeutic effects. 6-MP: 6-mercaptopurine; 6-TGN: thioguanine tiphosphate nucleotide; DHF: dihydrofolate; DHFR: DHF reductase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FPGS: folylpolyglutamate synthetase; GCPII: glutamate carboxypeptidase II; GMPS: guanosine monophosphate synthetase; Hcy: homocysteine; HPRT: hypoxanthine phosphoribosyl transferase; IMPDH: inosine-5′-monophosphate dehydrogenase; Met: methionine; MTHFD1: methylene-tetrahydrofolate dehydrogenase 1; MTHFR: MTHF reductase; MTR: methionine synthase; MTRR: MTR reductase; MTX: methotrexate; pgMTX: polyglutamated MTX; RFC1: reduced folate carrier 1; SAH: S-adenosyl homocysteine; SAM: S-adenosyl methionine; SHMT: serine hydroxymethyl transferase; TGMP: thioguanosine monophosphate; THF: tetrahydrofolate; TIMP: thioinosine monophosphate; TPMT: polymorphic thiopurine methyltransferase; TXMP: thioxanthine monophosphate; TYMS: thymidylate synthase.

are found to explain 41% variability in 6-MP induced toxicity cumulatively. Impaired intestinal absorption of folate (GCPII C1561T), lower circulating folate levels (RFC1 G80A) and lower 5-methyl tetrahydroflate (MTHFR C677T) might contribute towards impaired synthesis of SAM, which in conjunction with TPMT deficiency might contribute to hematological toxicity associated with high 6-TGN levels.

17.7 Association of genetic variants of folate pathway with methotrexate-mediated toxicity

Tantawy *et al.* (2010) reported association of MTHFR 677TT-genotype with grade III/IV myelotoxicity, increased relapse and decreased overall survival following MTX therapy. Chiusolo *et al.* (2012) showed inverse association of MTHFR A1298C polymorphism with hepatic and hematological toxicity in patients with lymphoproliferative malignancies, when they are treated with high-dose MTX and leucovorin rescue. Suthandiram *et al.* (2014) observed positive association of MTHFR 677TT-genotype with hepatic and hematological toxicity; and positive association of RFC1 G80A with hepatic toxicity in leukemic patients receiving MTX therapy. Liu *et al.* (2011) demonstrated lower hematological toxicity and lower MTX levels in patients with MTHFR 677C-1298C haplotype. Ando *et al.* (2013) observed lower levels of MTX polyglutamate in subjects with RFC1 G80A. Erculi *et al.* (2012) reported inverse association of MTHFD1 1958 G>A with hepatotoxicity while showing positive association of TYMS 5'-UTR 2R-allele with hematological toxicity following high dose MTX therapy. MTHFR 677TT-genotype was shown to increase MTX-mediated toxicity by 12-folds and had 7-year lower DFS (Angelo *et al.*, 2011). MTHFR 677TT-genotype was shown to be associated with decreased MTX clearance and increased incidence of mucositis, while MTHFR 1298C, RFC1 80G and TYMS 3R alleles showed lower risk for hematological toxicity (Faganel Kotnik *et al.*, 2011). MTHFR C677T and TYMS 5'-UTR 28bp tandem repeat are shown to be associated with MTX-mediated toxicity in children with ALL (Zgheib *et al.,* 2014). De Jonge *et al.* (2005) observed decreased *in vitro* sensitivity to MTX in patients with MTHFR 1298AC, MTRR 66 G-allele and cSHMT TT. Kishi *et al.* (2007) observed association of RFC1 G80A polymorphism with infection and gastrointestinal toxicity in ALL patients during remission induction and consolidation phases of chemotherapy, respectively. Lower MTX polyglutamate levels (RFC1 G80A), lower MTHF (MTHFD1 1958 G>A), lower methyl THF (MTHFR C677T) and decreased thymidylate synthesis (TYMS 5'-UTR 28bp tandem repeat) might influence DNA synthesis and methylation, thus contributing to toxicity. On the contrary, MTHFR A1298C, which has no direct influence in regulating MTHFR activity, has no toxicity. Similarly cSHMT TT-genotype that induces futile folate cycle confers protection against toxicity. In Table 17.1 the association of folate pathway genetic polymorphisms with hematological toxicity is shown.

17.8 Association of folate pathway gene expression with methotrexate pharmacodynamics

Vazquez *et al.* (2013) demonstrated association between the sensitivity to MTX treatment and increased expression of folate mitochondrial enzymes SHMT2, MTHFD2 and RFC1. Surtees *et al.* (1998) observed lowest level of MTHF and SAM in cerebrospinal fluid during induction and consolidation phases of chemotherapy, respectively with evidence of demyelination in ALL patients. Vezmar *et al.* (2009) observed lower levels of 5-methyl THF and SAM in the CSF of ALL patients who received intrathecal MTX without folinic acid rescue. Kager *et al.* (2005) observed differential expression of RFC1, MTHFD1, MTHFD2, folylpolyglutamate

Table 17.1. Association of folate pathway genetic polymorphisms with hematological toxicity in leukemia[1].

Genetic variant	Locus	Impact
GCPII C1561T	11p11.2	Induces 6-MP mediated hematological toxicity.
RFC1 G80A	21q22.3	Lower level of MTX polyglutamate. Associated with hepatic toxicity following MTX therapy. Synergistic interaction with TPMT×12 or TPMT CTTAT haplotype to inflate 6-MP mediated hematological toxicity.
SHMT C1420T	11q22.3-q23	Synergistic interaction with MTHFR 1298AC and MTRR 66 AG/GG decreases *in vitro* sensitivity to MTX.
TYMS 5′-UTR 28bp tandem repeat	18p11.32 and 18p11.31	Associated with MTX-mediated toxicity in children with ALL. Synergistic interaction with TPMT CTTAT haplotype to inflate 6-MP mediated toxicity.
MTHFR C677T	1p36.3	Associated with lower TPMT activity. MTHFR 677TT and MTHFR 677CT/1298AC genotypes are associated with hepatic toxicity during the maintenance therapy of ALL. MTHFR 677TT genotype was reported to be associated with gradeIII/IV myelotoxicity, increased relapse and decreased disease free survival following MTX therapy. Associated with hepatic and hematological toxicity following MTX therapy.
MTR A2756G	1q43	Associated with attention-deficit hyperactivity disorder in survivors of childhood ALL in conjunction with MTHFR C677T and MTHFR A1298C.
MTRR A66G	5p15.31	Acts synergistically with MTHFR A1298C and SHMT C1420T in reducing *in vitro* sensitivity to MTX. Determinant of impaired bone mineral density in childhood ALL patients in conjunction with MTHFR C677T.
MTHFD G1958A	14q24	Inverse association with hepatotoxicity following high-dose MTX therapy.

[1] 6-MP: 6-mercaptopurine; ALL: acute lymphoblastic leukemia; GCPII: glutamate carboxypeptidase II; TPMT: thiopurine methyl transferase; MTHFD: methylene-tetrahydrofolate (MTHF) dehydrogenase; MTHFR: MTHF reductase; MTR: methionine synthase; MTRR: MTR reductase; MTX: methotrexate; RFC1: reduced folate carrier 1; SHMT: serine hydroxymethyl transferase; TPMT: thiopurine methyl transferase; TYMS: thymidylate synthase.

synthetase and GCH1 across different subtypes of ALL namely, B-lineage hyper diploid (BHD), non-hyperploid B-lineage (BNHD), ALL with E2A-PBX1 and TEL_AML1 gene fusions and T-lineage ALL (T-ALL) suggesting that outcome of MTX therapy might be influenced by these expression patterns.

17.9 Influence of folate pathway genetic polymorphisms on survivors of childhood acute lymphoblastic leukemia

MTHFR C677T and MTHFR A298C polymorphisms are shown to be associated with attention deficit hyperactivity disorder (ADHD) in survivors of childhood ALL (Krull *et al.*, 2008). In addition to MTHFR A1298C, MTR A2756G polymorphism was also reported to be associated with ADHD after childhood ALL therapy (Kamdar *et al.*, 2011). MTHFR C677T and MTRR A66G polymorphisms are suggested to be genetic determinants of impaired total body mineral density in childhood ALL patients (Te Winkel *et al.*, 2011). These anomalies might be due to lower MTHF and SAM levels as reported in the earlier section.

17.10 Conclusions

SAM potentiates TPMT activity and hence crucial determinant of 6-MP induced toxicity. GCPII C1561T, RFC1 G80A and MTHFR C677T are the genetic determinants that are associated with 6-MP mediated toxicity. MTX toxicity was found to be higher in subjects with RFC1 G80A, MTHFD1 G1958A, TYMS 5'-UTR 28bp tandem repeat and MTHFR C677T polymorphisms. Two polymorphisms, i.e. MTHFR A1298C and cSHMT C1420T, exhibited inverse association with MTX mediated toxicity. Folate pathway genes are found to be differential expressed in different subtypes of ALL thus explaining subtype-specific pharmacodynamic effects of MTX. The existing literature supports the notion, that genetic variants of folate pathway should be considered as pharmacogenomic determinants that dictate both therapeutic efficacy and safety association with 6-MP and MTX.

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18. The effects of coffee and tea on leukemia cells

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Abstract

Tea and coffee both contain components that can potentially be used as effective agents in the treatment of leukemia. Tea, *Camellia sinensis*, contains polyphenols and other catechins that induce cellular apoptosis in leukemia infected cells. Specifically the polyphenol found in tea is theaflavin and the catechin is called epigallocatechin-3-gallate. Coffee, *Coffea arabica* and *Coffea canephora*, contains a powerful diterpene, similar to the catechins found in tea. Specifically the diterpene found in coffee is called kahweol. The diterpene, like the polyphenols, also prompted the leukemia infected cells to undergo apoptosis. Kahweol also induced the leukemia infected cells to stop growing and replicating.

Keywords: apoptosis, polyphenol, catechin, theaflavin, diterpene, antioxidants

Key facts

- Besides water, the two beverages that are widely consumed the most all over the world are coffee and tea.
- Coffee and tea are known to be consumed for their caffeine content; however both beverages contain other ingredients beneficial to the health of consumers.
- Leukemia is cancer of the blood and is prevalent in almost 300,000 people in the USA.
- Cellular apoptosis is the ability of a cell to undergo 'death'.

Summary points

- A catechin found in green tea called epigallocatechin-3-gallate, significantly increased cell apoptosis by inhibiting the vascular endothelial growth factor in leukemia infected cells.
- Coffee contains the diterpene kahweol which induced anti-proliferation and apoptosis in leukemia cells.
- Mothers should avoid drinking too much coffee while pregnant, to decrease the chance of leukemia diagnosis in the new-borns.
- In a cohort study, adults who consumed coffee were found to be less at risk for acute myeloid leukemia than those who did not consume coffee.

Abbreviations

AML	Acute myeloid leukemia
CML	Chronic myeloid leukemia
ALL	Acute lymphocytic leukemia
CLL	Chronic lymphocytic leukemia
DMSO	Dimethylsulfoxide
EGCG	Epigallocatechin-3-gallate
EGC	Epigallocatechin
ECG	Epicatechin gallate
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

18.1 Introduction

Two of the world's most popular beverages consist of coffee and tea (Hatzold, 2012). These prevalent drinks come in a variety of flavours and are prepared in a plethora of ways. Both beverages are consumed many times throughout the day, some being more widely consumed in the morning and others more widely consumed in the evening (Hatzold, 2012). Important ingredients found in these refreshments include antioxidants, polyphenols, and diterpenes. Leukemia is cancer of the blood and affects many people worldwide (Anonymous, 2013). How do the active ingredients found in coffee and tea affect leukemia cells in the human body? Roughly 1/3 of Americans consume coffee or tea at least once a day. Leukemia affects nearly 300,000 people in the USA (American Cancer Society, 2014). How does consuming either coffee or tea affect someone who was recently diagnosed with leukemia or is in remission?

18.2 Leukemia

It is estimated that 310,046 people are living with leukemia or are in remission from leukemia in the USA, according to the American Cancer Society in 2014. Leukemia is a cancer that affects the body's ability to form blood and its components such as red blood cells, platelets, and white blood cells (Anonymous, 2013). Leukemia affects the red bone marrow and the lymphatic system. Red bone marrow is very important in homeopoesis, the process of new blood cell formation and takes place at the sites of red bone marrow.

Blood cells originate from blood stem cells in the bone marrow (Starr, 2013). There are three major types of blood cells produced by blood stem cells: red blood cells, platelets, and white blood cells (Starr, 2013). Red blood cells carry oxygen to numerous places all over the body; without red blood cells, or with irregular red blood cells, the body would not be able to efficiently deliver oxygen (Starr, 2013). Platelets are used by the body to form blood clots to slow down or completely stop bleeding; without platelets the body would be more prone to blood loss. White

blood cells are used by the body to fight infection (Starr, 2013). Blood stem cells produce both myeloid stem cells and lymphoid stem cells. Myeloid stem cells are the type of stem cells which can produce red blood cells and platelets or myeloblasts (Starr, 2013). Myeloblasts are immature cells that can produce a type of white blood cells called granulocytes. Lymphoid stem cells are the type of stem cells that produce white blood cells called lymphoblasts, which differ from the white blood cells called granulocytes (Starr, 2013).

There are many types of leukemia; some types are more common in children while others are more common in adults. The most common form of leukemia in children is ALL (Anonymous, 2013). Chronic types of leukemia are more common in adults than in children (Anonymous, 2013). There are four major types of leukemia: acute myeloid leukemia, chronic myeloid leukemia, acute lymphocytic leukemia, and chronic lymphocytic leukemia (Anonymous, 2013). The term 'acute' refers to a hasty progression while the term 'chronic' refers to a slow progression. The term 'myeloid' refers to the bone marrow creating abnormal myeloblasts (a type of white blood cell), red blood cells, or platelets (Anonymous, 2013). The term 'lymphocytic' refers to the type of blood cells being affected by the cancer, lymphocytes or white blood cells (Anonymous, 2013). Acute types of leukemia affect immature cells causing the disease to develop rapidly. Symptoms of acute leukemia include anaemia (a condition in which there is not enough red blood cells to carry an adequate supply of oxygen to organs and tissues), bleeding, fever, and swelling of the lymph nodes (Anonymous, 2013). If left untreated, immature leukemia cells will continue to divide in the bone marrow resulting in death. Chronic leukemia is a gradual onset and is rarely the actual cause of death (Anonymous, 2013). Chronic leukemia renders patients more susceptible to illness and weakens the body's ability to coagulate.

18.3 Coffee and tea

Coffee and tea are very common beverages found to be consumed all over the world. Both beverages are purchased and consumed by many people at many times throughout the day. Coffee is consumed either hot or cold by roughly one third of the people in the entire world. Coffee is consumed in amounts larger than any other beverage (Hatzold, 2012). Part of coffee's popularity can be due to its stimulatory effect produced by the active ingredient caffeine, found in the coffee bean. An average cup of brewed coffee contains about 200 mg of caffeine (Hatzold, 2012). Coffee comes from two major sources: the plant *Coffea arabica* and the plant *Coffea canephora* (Hatzold, 2012). The plant bears fruit similar to cherries and when the fruits are ripe they are picked from the plant. After harvesting the fruit, the cherries are dispersed all over the ground where the sun dries them. After drying in the sun for a day or two machines are used to remove the bean. The bean is then roasted and eventually packaged and shipped for wholesale (Hatzold, 2012).

In addition to coffee, tea is one of the world's most widely consumed beverages other than water (Graham, 1992). Tea is created from the plant *Camellia sinensis* (National Cancer Institute, 2010). When the tea plant is ripe, the leaves are handpicked and collected. The leaves are dried out in the sun and then rolled to release the excess moisture. The leaves are then left to oxidize

and ferment depending on the kind of tea. The leaves are then packaged and shipped (National Cancer Institute, 2010). Caffeine is also prevalent in tea, but the concentration (amount) is less than the concentration found in coffee. One cup of tea contains between 60 and 90 mg of caffeine (National Cancer Institute, 2010). There are a few types of tea, usually named by their colour and grade. The major types of tea are: white, green, and black (National Cancer Institute, 2010). White tea is the least processed and the most pure of all the teas. It is made from the first buds of the tea plant and is very delicate. Since the leaves are extremely fragile the tea is prepared at lower temperatures (National Cancer Institute, 2010). Green tea is the most popular type of tea. Green teas is made from the unfermented leaves of the tea plant, *Camellia sinensis*. The process involves quick drying of the tea leaves which limits the amount of tannins in the beverage (National Cancer Institute, 2010). It is very common for various flavours to be added to make the taste of green tea more appealing. Black tea is the most oxidized type of tea. Black tea is fermented more than green or white which gives it the distinct dark colour (National Cancer Institute, 2010).

18.4 Polyphenols

Polyphenols are a dietary source of antioxidants. Antioxidants are molecules that inhibit other molecules from oxidation. Oxidation of molecules can produce free radicals. Free radicals are unstable molecules that can cause a chain of reactions inside the body. These reactions can be very damaging to cells. Free radical damage to cells can lead to cancer. Polyphenols found in tea are catechins and theaflavins, commonly referred to as tannins. Many other foods and fruit juices contain antioxidants. A comparison between green tea, black tea, grape juice, prune juice, and pineapple juice was conducted in 1998 (Table 18.1). The results illustrated that the amount of polyphenols were highest in the various teas, the most being found in different brands of black tea (Vinson and Dabbagh, 1998). Between the green tea and the black tea however, the better antioxidant is found in green tea.

18.5 Black tea and induction of apoptosis

Apoptosis is the automated 'program' in a cell that promotes death. In other words, apoptosis occurs when it is time for a cell to die. A cell understands that it is prompted to die after it has

Table 18.1. Amount of polyphenols present in various beverages (Vinson and Dabbagh, 1998).

Beverage	Average phenol amount (μM)
Fruit juices	5,605
Green tea	13,750
Black tea	21,060
Oolong tea	15,875

been affected by a certain level of toxic agent or multiple toxic agents. Therefore, apoptosis is extremely important in regulating and developing tissues in homeostasis. Homeostasis is an important mechanism in actively regulating the human body. More importantly, apoptosis plays a huge role in eliminating cancer cells. Induction of apoptosis is one mechanism of therapy that can be used in patients with leukemia. Many foods and beverages contain ingredients that can induce apoptosis. One common beverage that includes ingredients that can induce apoptosis is tea. Of the tea's active ingredients, polyphenols have been known to inhibit the formation and growth of tumours (Kundu *et al.*, 2004).

One experiment conducted in 2004 illustrates the effects of polyphenols on human leukemia cells (Kundu *et al.*, 2004). Two human leukemia cells were tested, acute myeloblastic leukemia (HL-60) and chronic myelogenic leukemia (K-562). Popular brands of tea were used: green tea, black tea from Darjeeling and black tea from Assam. The tea was prepared by brewing 2.5 g of dry matter in 100 ml of boiling water for five minutes. The liquid was then evaporated and the remnants were mixed with a little water and 10% DMSO. Each human leukemia cell, acute myeloblastic (HL-60) and chronic myelogenic (K-562), was treated with different concentrations of the tea compounds.

The results illustrated that both black and green tea could contribute to the induction of apoptosis. More specifically, black tea extracts and the polyphenol theaflavin induced apoptosis. Upon examination of the two human leukemia cells under a microscope, a few morphological features of apoptosis were observed. Cell shrinkage and apoptopic bodies were detected (Kundu *et al.*, 2004). It is important to observe cell shrinkage because generally a cell will get larger before it multiplies. Shrinkage of a cell therefore, corresponds with cell death. In addition to cell shrinkage, chromatin condensation, another key feature in call apoptosis was also observed (Kundu *et al.*, 2004). Observed by other scientists conducting another experiment on the three stages of apoptopic nuclear condensation, chromatin condenses into a fragmented form (Toné *et al.*, 2007). Unlike chromatin condensation in mitosis of cells, the chromatin condensation in apoptosis is more condensed and packaged in apoptopic bodies rather than chromosomes (Kundu *et al.*, 2004). Fragmented DNA was taken from the treated cells and examined. The examination of the fragmented DNA showed a 'ladder' formation, which is a typical feature of cell apoptosis (Kundu *et al.*, 2004). An analysis of the cell cycle also displayed induction of apoptosis. Theaflavin and both of the black tea extracts found to increase the sub G1 phase of the cell cycle (Kundu *et al.*, 2004). This phase is present in cells that are getting prepared for cell apoptosis. The phase, sub G1, occurs before the G1 phase. The G1 phase of the cell cycle is the first of four phases. In the G1 phase the cell grows largely in size and starts synthesizing mRNA in preparation for mitosis (cell division). Apoptosis induction from the tea is beneficial to leukemia infected cells, because it increased sub G1 activity which prevents the infected cell from growing and reproducing. An analysis of a DNA content histogram showed loss of DNA in the human leukemia cells (Kundu *et al.*, 2004). Loss of DNA is another common feature of apoptosis in cells. Acute myeloblastic leukemia cells (HL-60) and chronic myelogenic leukemia cells (K-562) what were treated with different kinds of tea displayed the induction of apoptosis was activated through caspases. Caspases are enzymes that activate cell apoptosis. An increase in concentration of the tea extracts was found to increase the activation of caspases (Kundu *et al.*,

2004). Leukemia cells treated with different concentrations of tea displayed different expression levels of the Bcl-2 and Bax protein. The Bcl-2 protein inhibits apoptosis while the Bax protein accelerates apoptosis (Kundu *et al.*, 2004). A decrease in the expression of protein Bcl-2 and an increase in the expression of the Bax protein would be beneficial for cell apoptosis. However, the untreated control human cell did not display any significant changes in the levels of Bcl-2 and Bax proteins (Kundu *et al.*, 2004). This means only the tea extracts altered the expression of the Bcl-2 and Bax proteins in leukemia infected cells.

18.6 Green tea and chronic lymphocytic leukemia

CLL cells produce and release a substance called VEGF. The VEGF affects CLL B cells significantly by increasing the cells immunity to apoptosis, cell death. CLL B cells have been shown to not only spontaneously secrete VEGF, but to also have two receptors located on the CLL B cell for VEGF to bind to (Lee, 2004). Since VEGF inhibits cell death, the cell will continue to secrete VEGF which will potentially affect other cells.

One catechin component of green tea is EGCG. EGCG was found to significantly increase apoptosis or cell death. In eight of the ten CLL samples conducted, EGCG increased cell apoptosis (Lee *et al.*, 2004). EGCG was also found to suppress Bcl-2 proteins in CLL B cells. Similarly to the effects of black tea concentrations on AML cells and CML cells, the Bcl-2 protein expression was lowered (Kundu *et al.*, 2004). EGCG was also found to suppress the two VEGF receptors. The ECGC inhibits the cells to bind VEGF which would then allow the cell to undergo apoptosis. The results of the tested CLL cells convey that VEGF signalling plays a regulatory role in signalling survival signals in the CLL cells. It also illustrates that interrupting the signalling pathway would result in caspase activation and apparent cell death in leukemia cells (Lee, 2004).

An experiment testing VEGF and EGCG in CLL cells revealed a couple of important findings. The experiment revealed that both VEGF and VEGF receptors induced an increase in anti-apoptotic proteins which illustrates a link between VEGF and cell survival in CLL cells (Lee *et al.*, 2004). However, this experiment also revealed the catechin ECGC increased cell death in CLL cells by means of decreasing secreted VEGF (Lee *et al.*, 2004).

A study conducted on rat basophilic leukemia cells illustrated the effects of tea polyphenols (Matsuo, 1997). Rat cells treated with tea polyphenols EGCG, EGC, and ECG showed a decrease in histamine, which was found to influence cell apoptosis (Matsuo, 1997). Histamine is an important substance found in cells, that prevents monocytic cells from being able to undergo apoptosis (Pyzlak *et al.*, 2009). EGCG was determined to inhibit a large amount of histamine release in the treated rat basophilic leukemia cells (Matsuo, 1997). Other polyphenols found in tea were also able to inhibit histamine release. EGC and ECG limited the release of histamine, but not as significant as EGCG (Matsuo, 1997). Although this study was testing the effects of tea polyphenols on animals (rats), it can still be applied to humans. Human leukemia cells tested

with various concentrations of tea also illustrated that EGCG played an important role in cell apoptosis (Lee *et al.*, 2004).

18.7 Coffee diterpene kahweol and apoptosis

Coffee has been found to contain a diterpene called kahweol. This diterpene is specific to the coffee bean and it has been reported to inhibit tumour cell growth activity. Similarly to the polyphenols found in tea, kahweol also affects cell apoptosis. An experiment on the effects of kahweol in coffee on human monocytic leukemia cells was conducted. Kahweol was directly added to the cells, varying in concentration of kahweol, and the cells were analyzed (Oh *et al.*, 2009).

Kahweol was found to induce anti-proliferation and apoptosis in human leukemia cells (Oh *et al.*, 2009). Cell apoptosis was determined in the U937 human leukemia cell by using a flow cytometric analysis. The analysis revealed that a remarkable amount of cells exhibited an increase of the sub-G1 phase. The sub-G1 phase was found to be dependent on dose and time (Oh *et al.*, 2009). Similarly to the black tea, an increase in concentration displayed an increase in the sub-G1 phase which is a good indicator of apoptosis (Kundu *et al.*, 2004). The analysis also revealed that the cells had an increase of DNA fragmentation. An increase in the concentration of kahweol increased the amount of fragmented DNA found in the cell. An increase of DNA fragmentation is another indicator of cell apoptosis. DNA fragmentation was also found to be increased in the CML and AML human leukemia cells treated with different concentrations of black tea extracts (Kundu *et al.*, 2004). The mechanism for kahweol-induced apoptosis was found to be by the caspase-3 inhibitor (Oh *et al.*, 2009). In an analysis of green tea extracts on human leukemia cells, the caspase-3 inhibitor was also affected (Lee *et al.*, 2004). Black tea extracts were also found to affect the caspase-3 inhibitor (Kundu *et al.*, 2004). An analysis of anti-apoptotic proteins was performed to determine more details of the mechanism kahweol has on apoptosis. The protein levels of Mcl-1, Bcl-2, Bcl-xL, and XIAP were found to be dramatically reduced depending on the concentration of the diterpene kahweol (Oh *et al.*, 2009). Overall, decreasing the presence of any of these four proteins would enhance cell apoptosis.

It has been widely accepted that the mitochondria organelle plays an important role in the death of a cell. A few events take place at the mitochondria that are fundamental in the process of apoptosis. Altered electron transport, changes in cellular oxidation-reduction reactions and release of caspase activators in mitochondria affect cellular apoptosis (Green and Reed, 1998). Altered electron transport and decrease in oxidation-reduction reactions disrupts function of the mitochondria. One caspase that is released in the mitochondria is cytochrome c. Cytochrome c is associated with cell apoptosis as an intermediate and the partial control of the death of a cell. Cytochrome c can disrupt the electron transport chain in the mitochondria or it can activate another caspase that destroys the cell from the inside (Green and Reed, 1998). Upon examining the cells treated with various concentrations of kahweol, an induction of cytochrome c was determined to be present in the cell cytoplasm (Oh *et al.*, 2009). This articulates that the

diterpene, kahweol, found in coffee causes mitochondria to release cytochrome c which is heavily involved in the process of cell apoptosis.

The effects of the diterpene kahweol found in coffee include anti-inflammation, anti-carcinogenic, and anti-tumour. Human leukemia cells treated with different concentrations of kahweol brought insight on how the cellular apoptosis mechanism functions in human monocytic leukemia cells (Oh *et al.*, 2009). Kahweol was found to activate the caspase-3 which induces apoptosis. Kahweol was also found to decrease the protein levels of Mcl-1, Bcl-2, Bcl-xL, and XIAP which are key to enhancing the effects of cellular apoptosis. Finally, kahweol was found to activate the mitochondrial substrate called cytochrome c, which produces important functions for preparing apoptosis (Oh *et al.*, 2009). Kahweol could potentially be isolated and used as a chemotherapy or other therapeutic source for individuals with leukemia or other cancer.

18.8 Maternal coffee consumption

Coffee, one of the world's most popular drinks, is consumed by many including those who are pregnant. How does drinking coffee affect the foetus and to what degree? A study from 2005 illustrated a positive association with maternal coffee drinking and acute leukemia (Menegaux *et al.*, 2005). There were 280 cases and 288 controls. The mothers of the cases and controls were questioned about their coffee, tea, and cola drinking habits during pregnancy. The odds ratio increased as the amount of coffee consumed increased. Daily intakes of three cups of coffee or less displayed an odds ratio of 1.0 (Menegaux *et al.*, 2005). Mothers who drank three cups of coffee or less daily showed no increase of leukemia diagnosis in their children. Daily intakes of four to eight cups of coffee displayed on odds ratio of 2.1 (Menegaux *et al.*, 2005). Pregnant women who daily drank between four and eight cups of coffee showed two times more likely diagnosis of leukemia in their children. Daily intakes of more than eight cups of coffee displayed an odds ratio of 2.8 (Menegaux *et al.*, 2005). Pregnant females who drank more than eight cups of coffee a day had almost three times more likely diagnosis of leukemia in their children. There was no association found between drinking soda or tea and childhood leukemia (Menegaux *et al.*, 2005). Based on these results, pregnant women should limit the amount of coffee they drink to no more than three cups a day.

18.9 Acute myeloid leukemia and coffee consumption

AML is more prevalent in adults than in children. A USA cohort study conducted from 1995-2003 displayed the effects of diet and lifestyle had on AML (Ma *et al.*, 2010). Specifically one of the target categories was coffee consumption. There were a total of 338 incident cases of AML. 294 cases drank coffee; 104 consumed 500 g or less a day, 130 consumed between 500 and 1000 g a day, and 60 drank 1000 g or more a day (Ma *et al.*, 2010). It was determined that those who did not consume coffee had a higher risk for AML (Ma *et al.*, 2010). Other factors including smoking

habits, age, ethnicity, eating habits, and exercise habits were also addressed. These factors also affect the risk of AML besides just coffee consumption.

18.10 Summary

Coffee and tea both contain important characteristics that either prevent or alleviate leukemia. Tea contains theaflavin and VEGF which were scientifically illustrated to induce cellular apoptosis in human leukemia cells. Coffee contains a diterpene called kahweol that was also proven to induce cellular apoptosis in human leukemia cells. No research has determined that extracting the active ingredients and supplementing it to patients diagnosed with leukemia is a viable cure. Further investigation and research is required, however it is illustrated that both of these beverages contain powerful ingredients that may aid in the cure for various types of leukemia.

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19. Nutrition for patients with thalassemia

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Abstract

Over the past few decades, the role of nutritional status in the health of patients with thalassemia (Thal) has gained attention of clinicians and scientists. Depressed circulating levels of vitamins C, D and E, as well as important essential trace minerals zinc and copper, have been observed. Growth problems are frequent in younger patients with thalassemia, in which the role of nutritional adequacy in relation to higher energy demands requires careful analysis to distinguish it from etiologies, such as chronic anemia and endocrine insufficiency. There is evidence that patients with Thal have increased need for certain nutrients due to poor nutrient absorption, elevated losses or increased nutrient turnover. Moreover, these nutrients have been shown to be involved in the comorbidities associated with Thal: growth failure, glucose intolerance, cardiomyopathy and low bone mineral density, which may be avoided or ameliorated with attention to improved nutritional status. Unfortunately, there are few well-designed, adequately powered studies of the role of general dietary or specific micronutrient intake in the cause, treatment, or prevention of the growth, body composition, and pubertal abnormalities seen in infants and children with Thal in medical settings, particularly of developed nations. Future research should consider nutritional supplementation for health outcomes in patients with both transfusion-dependent and non-transfused patients with Thal. Until these data are gathered, it is suggested that both pediatric and adult patients be monitored frequently and nutritional deficiencies corrected when observed in order to improve the overall health and quality of life in patients with Thal.

Keywords: thalassemia, hemoglobinopathy, nutrition, growth, antioxidants

Key facts

- Nutritional deficiencies in thalassemia are multifactorial and having an experienced nutritionist able to identify patients at risk is key to optimal clinical care.
- Children who are being monitored prior to placement on chronic transfusion therapy are especially vulnerable to nutrition and growth concerns.
- Vitamin C has a complex role in thalassemia that includes modulation of chelator ef cacy, which may change depending on the degree of iron overload.
- Patients with thalassemia are at particular risk for vitamin D deficiency; vitamin D deficiency has been associated with low bone mass.
- Zinc, the most well studied micronutrient in thalassemia, may be beneficial for growth, bone health and improving glucose tolerance for those at greatest risk.

Summary points

- The role of nutrition for overall health of patients with thalassemia has gained attention of clinicians and scientists over the past few decades.
- There is evidence that patients with thalassemia have increased needs for some nutrients due to either inadequate intake, increased nutrient turnover, and/or elevated nutrient losses.
- Resting energy expenditure in Thal is increased as hemoglobin level falls, which can be an important drain on growth calories in inadequately transfused patients.
- Nutrients commonly found to be deficient in patients with thalassemia include vitamins C, D, and E as well as important trace minerals, zinc and copper.
- Optimal nutritional status has been shown to be important for improved growth, muscle mass, energy expenditure, glucose homeostasis, and bone mineral density.
- Chelation is the strongest antioxidant in thalassemia, though antioxidants are important, they should not substitute for effective chelation therapy.
- Correction of nutritional deficiencies – macronutrient and micronutrient, should take precedence over high dose antioxidant supplementation.
- Both pediatric and adult patients should be monitored frequently and nutritional deficiencies corrected when observed in order to improve the overall health and quality of life in patients with thalassemia.

Abbreviations

BMD	Bone mineral density
BMI	Body mass index
DRI	Dietary reference intake
Hb	Hemoglobin
IGF-1	Insulin-like growth factor-1
Kcal	Kilocalories
REE	Resting energy expenditure
Thal	Thalassemia

19.1 Introduction

Thals are a heterogeneous group of inherited defects of synthesis of the globin proteins that make up Hb, leading to chronic anemia from ineffective hematopoiesis and hemolysis. Thal has a high prevalence in Mediterranean and Asian populations, with carrier rates as high as 60% in some regions of southeast Asia, and has been increasing in incidence in the USA due to recent immigration patterns (Vichinsky *et al.*, 2005; Weatherall and Clegg, 2001). Thals are classified as alpha or beta according to the globin chain that is deficient. While the carrier states are asymptomatic, homozygotes have anemia that can be severe enough to require regular blood transfusions for survival. Ineffective erythropoiesis is the hallmark of the disease with a hyperactive bone marrow but inadequate formation of mature red blood cells. The term 'thalassemia' mostly refers to patients who are dependent on regular transfusions. Patients with less severe anemia who do not require chronic transfusions are categorized as 'thalassemia intermedia'. Iron overload from transfused red blood cells is a universal complication in Thal major, but increased gastrointestinal iron absorption also leads to iron accumulation in non-transfused patients. The focus of this chapter will be on nutritional problems in patients with Thal major but may also be relevant to patients with Thal intermedia.

19.2 Why are patients with thalassemia at risk for nutritional deficiencies?

The etiology of nutritional inadequacy is multifactorial in Thal, and will be elucidated in detail with respect to specific essential nutrients later in the chapter. In general, the environment in which nutritional deficiencies develop is somewhat different for pediatric than adult patients with Thal, though for both groups, food intolerances (e.g. lactose intolerance), cultural food limitations, and inaccurate diet information may all contribute to poor nutrient intake.

A poor rate of growth is a hallmark of undernutrition and the need to address growth concerns is frequent in pediatric patients with Thal. Anemia is known to contribute to poor appetite (Mann, 1999), and the slower rate of growth may also be related to higher rates of REE (and thus higher calorie/kg need) and a low Hb level in the days preceding routine transfusions. When Hb falls

substantially below 9 g/dl, the effect of anemia on growth can be significant. This can result from unavailability of blood or from transfusion practices adopted by the medical team. Children who are being monitored for deciding if they should be placed on chronic transfusions often go through a period of moderate anemia (Hb: 5-8 g/dl) and are especially vulnerable to growth deficit. In toddlers and young children especially, parents often struggle with balancing the child's food preferences with worry about adequate nutrition. When tension regarding the iron content of foods is added to this dynamic, feeding difficulties can develop leading to lower calorie intake. Advice to use tea as the beverage at meals to lessen absorption of iron from food may result in tea replacing milk as the child's usual beverage. Thus, they will miss the calories, protein, calcium, zinc and other nutrients found in milk.

In adults, one of the primary areas of concern is optimization of nutrient density. Many patients have short stature and require fewer total kcal; therefore, their typical dietary intake may not contain adequate nutrient density (Fung *et al.*, 2012). For some patients, nausea or vague gastrointestinal complaints accompany the use of oral chelators and may lead to some missed meals, food intolerance and decreased food intake. There is a nutritional cost of the accelerated rate of red blood cell production in Thal, requiring increased amounts of folic acid, vitamin B12, B6, protein, zinc and other nutrients used in erythropoiesis. Increased requirement for anti-oxidants, for example vitamins C and E, β-carotene, copper and zinc, is related to protection against the oxidative stress and inflammation resulting from iron toxicity.

Essential minerals may be lost along with iron during chelation therapy, thus increasing the requirement for these minerals. Though chelation adherence is very important to the overall health of the patient, time spent focused on chelation therapy may borrow from time spent in planning for meals, food shopping and meal preparation; moreover, anemia may lower energy and motivation for these tasks.

Over the past few decades, the role of nutritional status in the health of patients with Thal has begun to be explored. Specific nutrients have been shown to be involved in the comorbidities associated with Thal: growth failure, glucose intolerance, cardiomyopathy and low bone mineral density, may be avoided or ameliorated with attention to improved nutrition. The research associated with nutritional care of Thal will be addressed in this chapter.

19.3 Total energy intake in thalassemia: is it inadequate?

One of the first questions to address is, 'what are total caloric requirements for patients with Thal?' Common clinical findings in young patients with Thal are poor growth and delayed pubertal development (Caruso-Nicoletti *et al.*, 2004; De Sanctis *et al.*, 2013; Raiola *et al.*, 2003). Though growth rate in very young children may be normal, it starts to falter at the beginning of puberty, and both transfused and non-transfusion dependent patients with Thal rarely achieve their genetic potential for height. Scientists have shown that not all of the growth failure observed is related to hypogonadism and growth hormone deficiency (Caruso-Nicoletti *et al.*, 2004).

Therefore, if nutritional status is partly to blame for poor growth, a general assumption holds that total calories and protein are inadequate to meet the specific needs imposed by the disease. In these patients, is the nutrition-related growth failure related to decreased dietary intake, increased requirements, or some combination of both?

A group from Israel used indirect calorimetry to estimate REE, the amount of calories consumed when an individual is at rest (Vaisman *et al.*, 1995), in seven adult transfused patients with Thal. They found that energy expenditure decreased by 9% following a red cell transfusion (P=0.02), which was related to Hb concentration. Sohn and colleagues also observed exercise limitations in Thal major were related to the degree of anemia (Sohn *et al.*, 2013). Overall, the increased need for total kcal is thought to be related to increased protein requirements for red cell turnover, as well as increased cardiac output.

Few studies have explored dietary intake in large samples of contemporary patients with Thal (Fung, 2010; Fung *et al.*, 2012). The North American Thalassemia Clinical Research Network conducted a cross-sectional analysis of dietary intake in 221 adult and pediatric patients with a variety of Thal syndromes (Fung *et al.*, 2012). Results suggest that although patients with Thal have generally adequate and sometimes excess intake of macronutrients (kcal, fat and protein), intake of specific micronutrients, such as vitamin D, E, folate, calcium, and magnesium were lower than the DRI for healthy individuals without Thal (Fung *et al.*, 2012). To clarify, DRIs were developed as guidelines for healthy individuals free of illness. Using the DRI as the recommendation for patients with chronic illness is not always appropriate given the potential for increased nutrient requirements for some patient populations. Therefore, simple nutritional comparisons to DRI levels, though informative, may be limiting.

A few small studies sought to relate the commonly observed co-morbidities of stunting and wasting to inadequate intake (Fuchs *et al.*, 1996; Soliman *et al.*, 2004). Soliman and colleagues placed 15 nutritionally deplete Thal children (average age of eight years) on a high calorie diet (130-150% adequate) for 8 weeks. They observed two months of increased caloric intake resulted in significantly higher BMI (kg/m^2), skinfold thicknesses, mid-arm muscle circumference and IGF-1 (Soliman *et al.*, 2004). Fuchs *et al.* (1996) performed a similar study on toddlers with Thal residing in Thailand a decade earlier. Linear growth did not improve after their short caloric intervention, though differences in weight gain, as well as plasma zinc, alpha tocopherol and IGF-1 were observed. Taken together, the improvements in IGF-1 in both of these studies suggest that some of the growth impairment in young Thal patients may be partially corrected by addressing nutritional concerns.

As noted above, there are only a handful of studies which have investigated dietary intake in patients with Thal in comparison with functional outcomes (i.e. growth, weight gain), and the data are limited either by small sample size, or by the lack of corresponding circulating levels of nutrients in the patients. Additionally, these studies have been completed in patients residing in countries with documented malnutrition in healthy non-Thal cohorts.

Recently, our group assessed dietary intake in 50 patients with Thal along with corresponding circulating levels of specific nutrients (Fung *et al.*, 2014), to address the question, 'Is dietary intake sufficient to support adequate circulating nutrient levels?' Data from this pilot study found that circulating levels of key nutrients were depleted, e.g. vitamin D and zinc, while dietary intake was at or above recommended levels. These data suggest that dietary intake is insufficient to support circulating levels of nutrients in optimally transfused Thal patients.

19.4 Nutrients of particular concern

19.4.1 Water soluble vitamins

In addition to the potentially increased requirement for total kcal, patients with Thal may be at particular risk for deficiency of essential micronutrients. The clearest example of this is folate, a nutrient involved with nucleic acid synthesis during formation of red blood cells. Given its requirement for erythropoiesis, folate may be deficient with increased needs for red cell production observed in Thal. Additionally, it is now understood that intracellular folate is readily catabolized by ferritin (Suh *et al.*, 2001). Patients with Thal intermedia are particularly vulnerable (Vatanavicharn *et al.*, 1979), similar to other hemolytic anemias, to the development of megaloblastic crisis that will respond to folate supplementation (Jandl and Greenberg, 1959). Chronically transfused patients have lower prevalence of deficiency, with some reports finding levels that are similar to healthy individuals. Elevated homocysteine levels, a clinical sign of folate deficiency, have been reported (Ozdem *et al.*, 2008; Sherief *et al.*, 2014). It is standard practice to provide supplemental folate to patients with Thal, especially non-transfused patients (Table 19.1).

Another essential water-soluble vitamin, ascorbate or vitamin C, is frequently deficient in patients with Thal (Elalfy *et al.*, 2013; Ray *et al.*, 1999). Vitamin C serves an important antioxidant function within the cell, has very strong reducing power (high redox potential) with the ability to regenerate via ubiquitous reductants such as glutathione, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate. Owing to its ability to reduce Fe^{3+} to Fe^{2+}, vitamin C is essential for both non-heme iron absorption as well as in the mobilization of iron from tissues (Nienhuis, 1981). Vitamin C modulates iron metabolism by stimulating ferritin synthesis, inhibiting lysosomal ferritin degradation, and decreasing cellular iron efflux (Lane and Richardson, 2014). On the other side, vitamin C is also important in facilitating release of stored iron in reticuloendothelial system and, probably plays a significant role in the uptake of non-transferrin bound iron. Thus, vitamin C has a complex role in Thal, which may change depending upon the degree of iron overload and non-transferrin bound iron present in the body. In patients with Thal, plasma vitamin C levels progressively fall with rising liver iron concentration (Elalfy *et al.*, in press; Fung *et al.*, 2014), likely reflecting greater turnover from oxidative stress. In extreme iron overload situations (liver iron concentration >25 mg/g), nearly all patients exhibit low plasma vitamin C levels. The vital role of vitamin C in mobilization of stored iron is evident during chelation therapy with deferoxamine. Deficiency has been associated with ineffective chelation (Chapman *et al.*, 1982; Elalfy *et al.*, in press; Wapnick *et al.*, 1969), while

Table 19.1. Nutrients of concern for inadequacy in thalassemia.

Nutrient group	Specific nutrient	Clinical signs observed
Macronutrients:		
Kilocalories	poor dietary quality/ nutrient density	growth failure
Micronutrients:		
Fat soluble vitamins	vitamin D	reduced bone mineral density
	vitamin K	
	vitamin E	increased oxidative damage
Water soluble vitamins	vitamin C	increased oxidative damage, decreased chelator ef cacy
	folate	worsening of anemia
Minerals:		
	zinc	poor growth, delayed puberty, reduced bone density, glucose intolerance
	copper	anemia, neutropenia, reduced bone density (severe)

supplementation can double the amount of iron excretion (Pippard *et al.*, 1982). The influence of vitamin C on iron excretion by oral chelators is not clear. Ascorbate supplementation in an animal model improved response to deferasirox (Brewer *et al.*, 2012). A recent randomized controlled trial in 180 transfusion dependent Thal children found 100 mg/day of ascorbate in addition to chelation therapy for one year reduced liver iron concentration by 10% above the control group (Elalfy *et al.*, in press). As a result of these observations, it is suggested that any patient with an unsatisfactory response to chelation should be tested for vitamin C sufficiency. However, high doses of vitamin C (>250 mg daily) are discouraged even in the setting of deficiency. In extremely iron overloaded patients, regular chelation should be established prior to beginning supplementation with vitamin C. In general, all patients should receive 60-80 mg as daily supplement in the form of a multivitamin pill.

19.4.2 Fat soluble vitamins

As a group, fat soluble vitamins appear to be of particular concern for deficiency in patients with Thal, though overt fat malabsorption is rarely observed (Montalto *et al.*, 1997). Vitamin D deficiency has received much attention in the scientific literature recently and in 2011, the Institute of Medicine released the new dietary guidelines for vitamin D (Institute of Medicine, 2011). This was followed by guidelines from the Endocrine Society which recommend even higher daily amounts of vitamin D for the general population and added considerations for additional requirements to consider in patients with some medical conditions or treatments (Holick *et al.*, 2011).

Patients with Thal are at particular risk for vitamin D deficiency. 82% of a sample of 361 patients with Thal residing in North America had insufficient levels of 25-OH vitamin D (defined as <30 ng/ml) (Vogiatzi *et al.*, 2009). Other international reports have found similar levels of inadequacy (Napoli *et al.*, 2006; Soliman *et al.*, 2008; Tantawy *et al.*, 2008). Vitamin D has many unique hormonal functions; its active form, 1,25-OH vitamin D has multiple roles in the body not only related to bone health and fracture reduction, but also cardiovascular health, immune function, blood pressure and cancer prevention (Institute of Medicine, 2011). Wood *et al.* found a weak though significant correlation between 25-OH vitamin D levels and left ventricular ejection fraction in patients with Thal, r^2=0.35 (Wood *et al.*, 2008). In this small study, all four subjects with dysfunctional ejection fractions (left ventricular ejection fraction <57%) also had deficient levels of vitamin D. However, when these researchers studied a larger more diverse group of patients, they found no association between vitamin D deficiency and cardiac dysfunction (Noetzli *et al.*, 2011). In multivariate analysis, another group found parathyroid hormone (PTH) levels to be the major predictor of increased myocardial iron in beta-Thal major patients (Dimitriadou *et al.*, 2010).

More recently, vitamin D receptor gene Fok-I polymorphisms have been associated with increased renal dysfunction in transfused patients with Thal. Dimitriadou *et al.* tested 34 transfusion dependent patients, of which, 12% were homozygous for the f allele of the Fok-I gene which was significantly correlated to proteinuria as well as hypercalciuria (Dimitriadou *et al.*, 2011). Elevated urinary calcium excretion may be partially responsible for the strong association between vitamin D deficiency and low bone mass. Patients with Thal who have deficient levels of vitamin D have a ten-fold greater risk of low bone mass after controlling for age, weight Z-score and hypogonadism. (Vogiatzi *et al.*, 2009).

Given the high prevalence of vitamin D insufficiency and potential for co-morbidities in Thal, vitamin D status should be monitored carefully in those patients who reside in Northern latitudes, are naturally dark skinned, who customarily shroud themselves, are liberal sun-screen users, have limited exposure to sunlight during mid-day hours or who have insufficient dietary intake of vitamin D due to lactose intolerance. Given vitamin D is a fat-soluble vitamin and stored primarily in the liver, it can be provided in large, infrequent doses to improve adherence. For transfused patients with 25-OH vitamin D levels <20 ng/ml, a 50,000 IU vitamin D_3 oral dose provided every 3-4 weeks at time of transfusion is suggested (Fung *et al.*, 2011). D3 has a longer half-life than D2 therefore can be provided to patients less frequently (Armas *et al.*, 2004). For patients with poor response to supplementation, a higher more frequent dosing may be required. Given the seasonal variability in vitamin D levels (Holick *et al.*, 2007), biannual monitoring is suggested to maintain vitamin D adequacy.

Vitamins A and E, considered non-enzymatic antioxidants, have also been reported to be deficient in Thal (Behera *et al.*, 2014; Claster *et al.*, 2009; Walter *et al.*, 2006), albeit less frequently than vitamin D. Vitamin E prevents the propagation of lipid peroxidation, and has been shown to help stabilize the red cell membrane. Vitamin E deficiency enhances red cell susceptibility to peroxidative damage (Chiu *et al.*, 1982). Vitamin A is essential for vision, growth, hematopoiesis

and immune function. Several studies have suggested that chronic transfusion therapy, though reducing the amount of hemolysis in these children, may actually increase oxidative stress and decrease antioxidant activity as result of iron overload (Behera *et al.*, 2014; Claster *et al.*, 2009; Walter *et al.*, 2006). Retinol concentrations one half the level of healthy controls and gamma-tocopherol one-fifth have been observed in young children with Thal (Behera *et al.*, 2014; Kassab-Chekir *et al.*, 2003). Serum levels of vitamin E are inversely correlated with ferritin (P=0.003) and liver transaminases, suggesting antioxidant consumption is related to iron overload (Livrea *et al.*, 1996). This has led scientists to explore the effects of vitamin E supplementation on iron overload-induced oxidative stress. Groups lead by Pfeifer and Tsoriere independently observed that supplementation with 400 to 600 IU of vitamin E for 3 months in patients with either Thal intermedia or E-beta Thal reduces oxidative stress in both red and white cells (Pfeifer *et al.*, 2008; Tesoriere *et al.*, 2001) and improves red cell susceptibility to peroxide-induced hemolysis (Suthutvoravut *et al.*, 1993). Reduced platelet reactivity has also been observed in adult Thal subjects (Unchern *et al.*, 2003). This level of supplementation is 12-18× the recommended dietary intake of vitamin E for adults. Neither of these therapies has yet to become standard of care, again, likely due to the paucity of robust large scale clinical trials in Thal, as well as the hesitation purposed by recent analyses which suggests that high dose vitamin E in animal studies may have a 'dark side', thus shifting our perception that antioxidants are always beneficial (Vrolijk *et al.*, 2015).

Vitamin K, the fourth known essential fat soluble vitamin has also been shown to be deficient in small numbers of transfused patients with Thal (Claster *et al.*, 2009). Vitamin K inhibits cardiac calcification and more recently has been shown to play a crucial role in decreasing bone loss of calcium, enhancing bone formation, and reducing fracture risk in healthy, non-Thal populations (Fujita *et al.*, 2012; Van Summeren *et al.*, 2009). Ozdemir and colleagues recently completed a pilot study in which they provided 50 μg vitamin K2 plus 200 IU vitamin D3 daily to 20 young (3-18 years) Thal subjects for one year (Ozdemir *et al.*, 2013). Spine BMD Z-score increased significantly from -1.2 to -0.8 (P=0.003), while there was a trend towards improvements in bone turnover markers. Future interventional trials with vitamin K are anticipated.

19.4.3 Minerals

Zinc is an essential trace mineral required for cell division, differentiation and gene expression. It is critical to the function of over 300 enzymes; as such it is important to a myriad of bodily functions including development and maintenance of the immune system, bone health, vitamin A metabolism, and actions of thyroid, insulin, testosterone and growth hormone. Zinc is a particularly important mineral to transfused patients with Thal because it is similar enough in size and charge to iron; therefore it has the potential to be chelated along with iron in those patients treated for iron overload.

Zinc is the most well studied of all the essential nutrients in Thal. Zinc deficiency has been documented in both transfused and non-transfused patients (Arcasoy and Cavdar, 1975; Kajanachumpol *et al.*, 1997) children and adults, residing in all areas of the world (Fung *et al.*, 2013; Sultan *et al.*, 2015). However, a minority have documented plasma zinc to be within the

normal range (Abbasinazari *et al.*, 2013; El Missiry *et al.*, 2014). The prevalence of low plasma zinc is reported to be between 20 to 80% of transfused patients with beta Thal (Bekheirnia *et al.*, 2004; Fung *et al.*, 2013). Depletion of circulating zinc may be due in part to the presence of proximal tubular damage (Cianciulli *et al.*, 1994) and hyperzincuria, as urinary zinc is elevated four-fold in patients with Thal compared to controls (Fung *et al.*, 2013; Uysal *et al.*, 1993). Increases in urinary zinc may be related to the presence or absence of diabetes, a co-morbidity present in roughly 20% of adult Thal and also associated with increased zinc losses (Al-Refaie *et al.*, 1994). Alternatively, chelators used to treat iron overload may place patients at risk for zinc deficiency (Maclean *et al.*, 2001). Roughly 20% of patients chronically chelated with deferiprone will develop a zinc deficiency if not prophylactically supplemented (Al-Refaie *et al.*, 1994).

Growth failure in Thal has been associated with depressed plasma zinc levels (Arcasoy *et al.*, 1987). Three decades ago, Arcasoy and colleagues were the first to give zinc supplement to young, regularly transfused, but non-chelated, patients with Thal. 21 patients received between 22-90 mg Zn/d over a period of 1-7 years, the remaining patients received only their usual transfusion regimen. Though only 50% of the sample were considered growth delayed at study initiation, yet they observed an increase in height velocity in the zinc-supplemented group ($P<0.01$) (Arcasoy *et al.*, 1987).

Abnormalities of zinc metabolism have also played a role in the pathology of osteoporosis in patients with Thal. BMD Z-scores are lower in adolescent males and females with severe zinc deficiency compared with Thal with normal serum zinc (Bekheirnia *et al.*, 2004; Shamshirsaz *et al.*, 2007). Between 2010 and 2013, our group conducted an 18-month long placebo controlled trial of zinc supplementation on bone health in 45 patients with Thal. Using intention to treat analysis, after adjusting for baseline and pubertal stage, patients provided 25 mg/d of zinc had significantly greater increases in whole body bone mineral content and density (Fung *et al.*, 2013). Moreover, hip and spine BMD Z-scores in the placebo group declined during the trial while for those in the zinc group, bone health stabilized.

Within the last few years, the relationship between zinc deficiency and diabetes in patients with Thal has been explored. Dehshal *et al.* found that 37% of subjects with Thal had depressed serum zinc levels, which were associated with lower fasting and one-hour post-oral glucose tolerance test serum insulin concentrations (Dehshal *et al.*, 2007). More recently, our group observed similar results with abnormal glucose tolerance curves in Thal patients with low zinc levels (Fung *et al.*, 2015). These data support the hypothesis that zinc deficiency in Thal is associated with pancreatic insufficiency and decreased glucose disposal.

Though zinc supplementation is still far from standard of care, it is the most well studied of all the nutritional supplements in Thal, and may be beneficial for growth, bone health and improving glucose tolerance for those at greatest risk. For those with low serum zinc at risk for zinc deficiency, a 25 mg Zn supplement should be considered. During supplementation, serum copper must also be monitored, and kept above 70 μg/dl.

Until recently, copper was an overlooked trace element for patients with Thal. However, it has been known to be essential for decades due to its role in cytochrome c oxidase, which is required for cell respiration, it is found in many superoxide dismutases. Given it is nearly ubiquitous in the food supply, many thought it would be nearly impossible to become deficient in this essential mineral. With the exclusion of Menkes' disease, there are very few examples in clinical textbooks of copper deficiency, in the absence of high dose zinc supplementation. However, patients with Thal have a unique set of circumstances that set them up for deficiency. Copper deficiency can produce anemia, neutropenia, bone abnormalities, osteoporosis, and abnormalities in glucose metabolism. Depletion of circulating copper in Thal may be due in part to renal tubular excretion, increased losses with chelation as well as poor dietary intake. Foods rich in copper are also rich in iron and zinc. The prevalence of low copper levels is between 32 to 36% in adults (Banihashem *et al.*, 2013; Mashhadi, 2013). However, elevated levels are also observed, and are a marker of inflammation, observed in the face of very low serum zinc (Mashhadi, 2013). Therefore, in order to determine the legitimacy of serum copper in Thal, that it be assessed along with its transport protein, ceruloplasmin. When both are low, a deficiency is near certain.

From the published studies highlighted herein, it is clear that patients with Thal frequently have depressed circulating nutrient levels, the potential for increased requirements of specific nutrients and some indication that dietary intake is inadequate. The etiology for these presumed nutritional deficiencies are myriad (Table 19.2).

Table 19.2. Etiology of nutritional deficiency in thalassemia.

Intake	Expenditure
Inadequate dietary intake due to poor appetite	Increased mineral losses due to non-specific chelation
Inadequate or inappropriate intake due to feeding behavior issues in toddlers and young children	Essential trace mineral sequestration (Zn, Cu) in the liver due to iron-overload
Insuf cient nutrient density	Increased non-transferrin bound iron leading to increased oxidative stress and antioxidant consumption
Lactose intolerance	Ineffective erythropoiesis and increased cardiac output leading to elevated energy expenditure
Avoidance of foods rich in iron often limits protein intake	
Replacement of nutrient dense beverages with tea	
Nausea, cramping from use of oral chelators leads to missed meals, specific food intolerances and decreased food consumption	

19.4.4 Antioxidants

A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans. The increased production of pro-oxidants in patients with Thal is well established, and cellular changes that result from oxidative stress well documented, such as increased lipid peroxidation (Walter *et al.*, 2006), protein oxidation (Trombetta *et al.*, 2006) and DNA oxidation. Some antioxidants we have described already, vitamins A, C, E and zinc. Others are just now becoming more understood for their role in improving red cell characteristics and reducing transfusion requirements and such as lycopene, carnitine and curcumin; however they are beyond the scope of this review.

19.5 Iron overload in thalassemia: should dietary iron be limited?

Given the relationship between iron overload and organ dysfunction in Thal, counseling patients to consume a diet low in iron has been part of the standards of care for decades. Typically, a diet that is low in iron rich foods such as red and organ meats, and fortified breakfast cereals is recommended for all patients. However, there is debate regarding the effectiveness of reducing dietary iron consumption for the transfused subject. The amount of iron obtained from just one unit of packed red blood cells (200 mg) far outweighs the amount of iron obtained from a 3 oz steak (5 mg). Typical daily iron accumulation from transfusion related iron is ~20 mg/day (two units every three weeks), compared to iron accumulation from an iron rich diet: ~4 mg (assuming 30% absorption). A low iron diet may decrease the quality of life in some transfusion dependent patients and or create a false sense of security, that is, if they decrease their dietary iron intake, they may need to be less diligent with regard to chelator adherence. Moreover, iron absorption from the intestinal tract is increased in inadequately transfused patients due to effect of hepcidin on iron homeostasis (Ganz and Nemeth, 2012).

However, for the non-transfusion dependent patient with Thal, reducing iron in the diet is an essential part of nutritional counseling as these patients have a tendency for increased absorption of iron from the intestinal tract. Black tea has been shown in one study from 1979 (n=6 subjects) to reduce the absorption of dietary iron from plant based foods up to 95% (De Alarcon *et al.*, 1979).

19.6 Nutritional guidelines for thalassemia

Given these recent findings of inadequate nutrition in children and adults with Thal, careful nutrition evaluation is an essential component of comprehensive care. As we have elucidated throughout this chapter, nutritional deficiencies are multifactorial, and having an experienced nutritionist able to identify risk factors in patients is key to optimal clinical care. Part of the overall nutrition evaluation might include height, weight, BMI, measures of skinfold thickness, whole body dual energy x-ray absorptiometry scan to assess body fat, evaluation of caloric requirements,

as well as a thorough diet and supplement history. A nutritional interview is important to assess nutrient intake and adequacy, including diets that may exclude foods based on ethnic preferences, religious constraints, or misinformation from the internet or hearsay. Discussion of recommended nutrition supplements is an essential part of the nutrition interview as self-selected choices may be of unbalanced or harmful composition. A discussion of energy intake is paired with a review of energy expenditure and the importance of exercise and appropriate sun exposure in improving bone health. A multidisciplinary assessment that involves nutrition, endocrine and hematology specialists is essential for addressing growth problems in Thal.

However, there are few nutrition consensus statements to suggest a standard of care for patients with Thal. Guidelines published by the American Academy of Pediatrics for children with Thal (Fung and Chung, 2014) as well as The Northern California Comprehensive Thalassemia Center (Vichinsky *et al.*, 2012) have put forth a set of nutritional monitoring recommendations which may provide a useful guide for clinicians (Table 19.3). More specifically, guidelines on the clinical management of Thal published in 2008 by the Thalassemia International Federation, while recognizing the risk of multiple nutrient deficiencies, do not recommend supplementation of any individual micronutrient other than folate. Rather, they stress a diverse, high vegetable and low simple carbohydrate diet to maximize ingestion of these nutrients and delay the onset of impaired glucose tolerance. Most experts agree that there should be close monitoring of the nutritional status of patients with Thal, with inclusion of a dietitian or nutritionist in the comprehensive care team.

Routine, longitudinal growth and nutritional status assessment is essential to care of patients with Thal. These data should inform a diagnosis of growth failure or malnutrition, and provide the basis for planning the nutrition intervention strategy. One of the barriers to adding high calorie oral supplements for patients is that many of them contain iron, so alternative nutrient dense strategies must be considered. Given the common occurrence of linear growth failure, the biological parental heights should be obtained, recorded on the patient's growth chart and used to assess the pattern of linear growth. It is important to remember that short stature is not a part of the genetic expression of Thal and with optimal nutritional intake, optimal transfusion therapy and control of iron burden most children will be able to grow to their genetic potential for height. For children over nine years of age, the progression through pubertal development should be evaluated and documented in the physical examination every 6 to 12 months. Levels of key circulating micronutrients should be assessed on an annual basis and corrections made when deficiencies are observed. Given the seasonal variability in vitamin D levels, and the extremely high prevalence of deficiency in Thal, it is suggested that 25-OH vitamin D be assessed every six months.

19.7 What is needed for future nutritional trials in thalassemia?

There are few well-designed, adequately powered studies of the role of general dietary or specific micronutrient intake in the cause, treatment, or prevention of the growth, body composition, and

Table 19.3. Suggested nutrition monitoring guidelines for patients with thalassemia.[a]

Growth/development	Sample type	Frequency	Adequacy
Weight, kg		Monthly (Tx[b]) Quarterly (non-Tx)	>10th and <90th percentile
Height, cm[c]			>10th and <90th percentile
Sitting height, cm		Every 6 months	
Calculate body mass index, kg/m^2		Every 6-12 months	>10th and <90th percentile
Calculate growth velocity, cm/year		Every 6-12 months	Infancy: 23-28 cm/year Childhood: 5-6.5 cm/year Puberty: 8.3 cm/year (girls), 9.5 cm/year (boys)
Puberty: tanner staging (starting at 9 years)		Every 6-12 months	
Nutritional laboratory[d]			
Albumin		Annually	3.5-5.0 g/dl
Folate[e]	Serum	Annually	>3 ng/ml
Vitamin D, 25-OH	Serum	Every 6 months	≥30 ng/ml
Vitamin C, ascorbate	Plasma	Annually or more frequently if unsatisfactory response to chelation	>0.4 mg/dl
Vitamin E, α and γ tocopherol	Serum	Annually	Age and gender dependent
Zinc	Serum, TE, F[f]	Annually	≥70 μg/dl
Copper	Serum, TE	Annually	≥70 μg/dl
Ceruloplasmin	Serum	Annually	Age and gender dependent
Other			
Bone densitometry		Annually, starting at 8 years	Z-score >-2.0
Review of dietary intake		Annually	

[a] Adapted from Vichinsky *et al.* (2012) and De Sanctis *et al.* (2013).

[b] Tx: transfusion-dependent patients.

[c] Adequacy of growth is dependent upon the child's genetic potential for growth determined by the mid-parental height index.

[d] For transfused patients, best to draw laboratory values prior to transfusion, (e.g. morning of).

[e] May be possible to use red blood cell folate in the non-transfused patient, as it is typically a better indicator of tissue stores than serum folate.

[f] TE: trace elements; F: fasted stated. All trace elements need to be collected into trace element-free vacutainers and it is important that these be collected in the fasted state.

pubertal abnormalities seen in infants and children with Thal in the medical settings, particularly of developed nations.

Measurable goals should be defined prior to any interventional study being conducted. Is the goal of the study simply to replace nutritional deficiencies that are defined by standards derived from healthy population? Alternatively, is the goal of the study to reduce or delay organ damage, lower oxidative injury to membranes, improve mitochondrial function, target organ damage, improve energy metabolism, improve immune function, reduce transfusion requirement or simply to stabilize the membrane of transfused red cells.

Will the study consider health outcomes for both patients with transfusion-dependent and non-transfusion dependent Thal? Will children and adults be considered? How about those who already have the co-morbidity of interest (e.g. diabetes, osteoporosis), will they be included? Particular attention should be placed on vitamin and mineral metabolism, drug nutrient interactions, adherence to the supplement in the face of the many health concerns and any disease-specific alternations that may be required (altered meal plan for diabetics). All of these questions should be considered carefully before an interventional study is undertaken.

In this organized set of patients, families, and community support groups, such research is feasible if funding is available.

19.8 Conclusions

Depressed circulating levels of key fat and water-soluble vitamins, as well as important essential minerals, are frequently observed in patients with Thal. There is evidence that these patients have increased needs for certain nutrients due to poor nutrient absorption, elevated losses or increased nutrient turnover. The contribution of poor nutrition towards the occurrence of frequently reported co-morbidities in Thal should always be considered. It is suggested that both pediatric and adult patients be monitored frequently and nutritional deficiencies corrected when observed in order to improve the overall health and quality of life in patients with Thal.

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20. A growing risk in a growing population: obesity's impact on leukemia incidence and survival

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Abstract

Rates of obesity have risen over the past half century and are now a nearly ubiquitous presence in the healthcare setting. As rising cancer rates paralleled the rise in obesity, epidemiologic studies established a conclusive link between obesity and cancer. As obese patients became increasingly common on cancer trials and in oncology practices, it became evident that obesity may influence not only cancer onset but cancer outcomes too. In this chapter, we review the epidemiologic linkages between obesity and the incidence of, and survival from, adult and pediatric hematologic malignancies. We then explore hypotheses describing plausible biologic connections between the two co-morbidities. Obesity-associated cancers and the associated adverse impact on survival constitute a significant public health concern requiring urgent intervention.

Keywords: weight, fat, body mass index, cancer, outcomes

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Key facts

- Obesity is prevalent at stable rates in the USA and high-income countries; the export of the 'Western diet' is resulting in similarly growing rates in the developing world.
- The American Cancer Society's Cancer Prevention Study II was a landmark study published in 2003 that conclusively demonstrated the presence of an association between obesity and cancer.
- As obesity became more prevalent in society, and thus the clinical setting, considerable variability in practice ensued among oncologists in adjusting treatment plans for obese patients.

Summary points

- Obesity in adults is associated with increased risk for overall cancer, and specifically for hematologic malignancies.
- Additional research is necessary to determine whether the effect on cancer incidence is: present across all ages and ethnicities; 'dose-dependent' or whether a threshold must be exceeded.
- Obesity is associated with poorer survival in adult and pediatric hematologic malignancies.
- Obesity is associated with distinct patterns of severe and/or fatal toxicity dependent on treatment regimen for the different hematologic malignancies.
- The pathophysiology of obesity and leukemia's interaction remains unknown, but may be due to pharmacokinetic and pharmacokinetic differences in the obese and/or mediated by adipokines and fat-related cell signaling.

Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BMI	Body mass index
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
IGF-1	Insulin growth factor-1
IL	Interleukin
JAK/STAT3	Janus kinase 2/signal transducer and activator of transcription 3
MAPK/ERK	Adenosine monophosphate-activated protein kinase/extracellular signal related kinases
OR	Odds ratio
PI3K/AKT/mTOR	PI3K-kinase/protein kinase B/mammalian target of rapamycin
RR	Relative risk
TNF-α	Tumor necrosis factor α
TRM	Treatment-related mortality
TRT	Treatment-related toxicity

20.1 Introduction

Obesity exhibits a pervasive influence on the human host via a multitude of known pathways and an equal number that remain as yet undiscovered. Nonetheless, by the very definition of the obese state, these pathways – both known and unknown – cause individual ripples that coalesce into a wave of unintended consequences on the body's immune system and its ability to maintain homeostasis. In fact, the Centers for Disease Control and Prevention in the USA defines obesity as the 'ranges of weight that are greater than what is generally considered healthy for a given height' and that have been shown to 'increase the likelihood of certain diseases and other health problems' (Centers for Disease Control and Prevention, 2015). Obesity has been associated with multiple comorbidities including cardiovascular disease (Wilson *et al.*, 2002), diabetes (Mokdad *et al.*, 2003), metabolic syndrome (Laurson *et al.*, 2011), non-alcoholic fatty liver disease (hepatic steatohepatitis) (Marchesini *et al.*, 2008), pancreatitis (Hong *et al.*, 2011), venous thromboembolism (Stein *et al.*, 2005), and infertility (Pasquali *et al.*, 2007). As described below, we are becoming increasingly aware that cancer too is not immune from obesity's impact on the body.

Over the preceding decades, obesity's adverse impact on human health has become evident due to the exponential increase in its prevalence; where as one adult in five was obese in the early 1970's, by the turn of the century this figure had increased to one in three (Flegal *et al.*, 2002; Wang and Beydoun, 2007). Despite public service campaigns and public health efforts to the contrary, obesity rates have remained plateaued for more than a decade (Flegal *et al.*, 2010). Naturally,

children and adolescents have also been affected with corresponding burgeoning rates of obesity (Ogden *et al.*, 2012). It is therefore perhaps not surprising that as the incidence of cancer grew over the years concurrently with obesity (Smith *et al.*, 2010), the question of a possible connection between the two became of interest. Likewise, as obese cancer patients became well-represented in clinical practices and on clinical trials, the issue of how obesity impacts cancer therapy became relevant. These two questions, and the hypothesized pathophysiology of any such interaction, remain a focus of investigation today as epitomized in statements by Harold Varmus, recent director of the National Cancer Institute of the National Institutes of Health, '[the] contribution that obesity makes to cancer [is a] very important feature of public health…', 'it's estimated that if there were no obesity in the USA, we'd have about 20% fewer cancer deaths in this country. And yet we don't really understand what the connection is between obesity and cancer' (Kaiser, 2011; Varmus, 2011).

20.2 Epidemiology of obesity, cancer, and hematologic malignancies

20.2.1 Obesity and cancer incidence

If obesity were to significantly intensify leukemogenesis (i.e. the development and progression of leukemic disease) as postulated, such an affect should be evident in studies at the population level. The first step was therefore to determine whether a relationship exists between obesity and the risk for developing cancer, including specifically hematologic malignancies. From the beginning, a combination of small registry studies and reports displayed a consistent pattern of association between obesity and increased risk for developing certain cancers (Bergstrom *et al.*, 2001; Giovannucci *et al.*, 1995; Hsieh *et al.*, 1990; Kaaks *et al.*, 2002; Moller *et al.*, 1994; Snowdon *et al.*, 1984; Wolk *et al.*, 2001). Nonetheless, despite the mounting preponderance of evidence, the limited nature of these studies precluded the definitive statement that 'obesity is associated with the risk for developing cancer'. In 2003, however, Calle *et al.* published in the New England Journal of Medicine the findings from an obese subset within the Cancer Prevention Study II, an immense prospective cohort study performed by the American Cancer Society that enrolled over a million participants (1,184,617) who were cancer-free in 1982. The study then followed them for 16 years and found a striking association between obesity in adults and a greater than 50% increase in the risk of dying from cancer, both site-specific and 'all cancers,' including hematologic malignancies. Moreover, maintaining a 'normal' weight was calculated to prevent one out of every five to six cancer deaths in men and women respectively. This landmark study conclusively established the linkage between obesity and cancer, and served as the foundation supporting the need for continued investigation.

Differences in the strength of association were noted across cancers in the cohort, thereby raising the importance of examining not only categorical trends, but also the risk derived from obesity in individual cancers. Within hematologic malignancies, Table 20.1 shows the results of over 40 case-control and cohort studies (inclusive of meta-analyses) exploring the question of obesity in adults and the incidence of hematologic malignancies, namely leukemia, lymphoma, and

Table 20.1. Obesity-associated risk for hematologic malignancy in adults.

Study (year)	Study design	Hematologic malignancy[1]	RR/OR[2] for incidence (95% CI)
Larsson *et al.* (2008)	Meta-analysis	ALL	1.65 (1.16-1.95)*
		AML	1.52 (1.19-1.95)*
		CLL	1.25 (1.11-1.41)*
		CML	1.26 (1.09-1.46)*
Castillo *et al.* (2012)	Meta-analysis	ALL	1.62 (1.12-2.32)*
		AML	1.53 (1.26-1.85)*
		CLL	1.17 (1.08-1.27)*
		CML	1.16 (1.04-1.30)*
Kasim *et al.* (2005)	Case-control study	ALL	1.90 (0.90-4.10)†
		AML	1.60 (1.20-2.20)†
		CLL	1.40 (1.00-1.80)†
		CML	2.30 (1.50-3.40)†
Strom *et al.* (2009)	Case-control study	CML	3.09 (1.56-6.13)†
Larsson *et al.* (2007)	Meta-analysis	NHL	1.20 (1.07-1.34)*
Larsson *et al.* (2011)	Meta-analysis	HL	1.41 (1.14-1.75)*
Willet *et al.* (2006)	Case-control study	HL	2.20 (1.10-4.30)†
Wallin *et al.* (2011)	Meta-analysis	MM	1.21 (1.08-1.35)*

[1] ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myeloid leukemia; NHL = non-Hodgkin lymphoma; HL = Hodgkin lymphoma; MM = multiple myeloma.
[2] * = risk ratio (RR), † = odds ratio (OR).

multiple myeloma. Obesity was found to consistently contribute an increased risk for developing leukemia across all adult leukemia subtypes (Castillo *et al.*, 2012; Kasim *et al.*, 2005; Larsson and Wolk, 2008). Of note, the study by Strom *et al.* assessed obesity and risk for CML at multiple ages as compared to the matched controls; the authors found a significantly greater – but not increasing – risk for CML at all time-points, thereby hinting that no additive effect from duration of 'exposure' to obesity was present (Strom *et al.*, 2009). Obesity was also associated with risk for developing other hematologic malignancies; it was associated with increased risk for lymphoma, both non-Hodgkin lymphoma overall and most sub-types, Hodgkin lymphoma, and multiple myeloma (Calle *et al.*, 2003; Larsson and Wolk, 2007, 2011; Wallin and Larsson, 2011; Willett and Roman, 2006).

The pattern, and strength of effect, did not differ significantly by sex, with both men and women at increased risk for a hematologic malignancy from being obese. The above studies provide strong support for the proposed connection between hematologic malignancies and obesity: they represent a substantial number of patients, are sourced internationally from diverse settings, and

most importantly, utilize uniform criteria to define obesity as established from the Centers for Disease Control and Prevention and WHO (BMI ≥30 kg/m^2). The studies also include in their design or analyses most historical predictors of risk as relevant to the specific malignancies, thus further supporting an independent link between obesity and malignancy.

Nonetheless, several key epidemiologic questions with potential to affect public health strategy remain surrounding this proposed linkage. Although the effect is present across different populations, it remains unknown whether obesity contributes to risk uniformly across different ethnicities and/or races. It is clear that rates of obesity differ sharply by ethnicity and race with greater prevalence of obesity (by 10-20%) in non-Caucasian populations and only a widening disparity over the past several decades (Wang and Beydoun, 2007). These same populations also demonstrate greater incidence of hematologic malignancies than Caucasians (Dores *et al.*, 2012; Matasar *et al.*, 2006; Yamamoto and Goodman, 2008). None of these studies on ethnicity and demographics included obesity as an environmental exposure. Additional research is thus needed to determine whether ethnicity's association with leukemia incidence may be related to rising rates of obesity within these same populations and/or whether a potential additive or synergistic effect is present between obesity and other potential environmental or genetic contributions to cancer initiation. Similarly, due to stark differences in biology between pediatric and adult hematologic malignancies, it is also unknown whether obese children are similarly at-risk, or even more intriguing/concerning, whether childhood obesity conveys risk for adult-onset of a malignancy. The only studies conducted in the pediatric age range to date focus on high birth weight infants, in effect reporting on *in utero* changes to growth factors and hormones as a leukemia-initiating event (Caughey and Michels, 2009; Hjalgrim *et al.*, 2003). Informal examination of large prospective clinical trials in pediatric ALL and AML reveals the prevalence of obesity to be relatively similar to that of the general population (Gamis *et al.*, 2014; Hijiya *et al.*, 2006; Orgel *et al.*, 2014a). Only in one pediatric AML study was obesity over-represented with a higher prevalence more akin to adult populations (Inaba *et al.*, 2012). Yet, no reported data to date specifically examines the influence of obesity in childhood on incidence of leukemia in pediatric or late-onset adult populations. The other outstanding question that must be answered to effectively guide research and policy is whether obesity exerts a dose-dependent (i.e. increasing weight related to increasing risk) or threshold effect (i.e. only those over a certain extreme of body fat are affected) on risk for malignancy. While some studies found a dose-dependent effect (Calle *et al.*, 2003; Larsson and Wolk, 2008, 2011), others found only an effect in the obese and not in those overweight (BMI 25-29 kg/m^2) (Castillo *et al.*, 2012). Additional research is therefore necessary to determine if greater body fat even below the threshold for obesity remain harmful.

Epidemiological evidence strongly supports the proposed association between obesity and risk for cancer, including specifically hematologic malignancies. Yet, answers are still needed as to the nature of this relationship. The obese state is not static over years; body fat in individuals varies constantly with lifestyle changes. Identifying key demographic populations at greatest risk and establishing weight or body fat targets to reduce the risk for developing a malignancy will serve as the basis for mitigating this aspect of obesity's impact on public health.

20.2.2 Clinical practice patterns for obesity during therapy

Obesity not only affects the risk for developing leukemia, but also the leukemia therapy itself. The increasing prevalence of obesity raised multiple clinical concerns from treating oncologists on whether, and how, to modify therapy in the obese to maintain appropriate and safe chemotherapy dosing. Since the advent of effective chemotherapy for cancer treatment in the 1960's, chemotherapy has been dosed as a reflection of body weight (via calculated body surface area) (Freireich *et al.*, 1966). In extreme obesity, providers therefore became alarmed at the resultant high calculated doses of chemotherapy. As obesity affects multiple end-organs, it was also unknown whether additive toxicity would result from further insult due to hepatotoxic and nephrotoxic chemotherapy. As such, a wide range of practices developed wherein patients were treated with 'full-dose' chemotherapy based on actual weight, empirically 'dosed-reduced' per calculated ideal body weight, or artificially capped according to the physician or pharmacist's comfort level at an unofficially 'accepted national standard' of 2.0 m^2 (Field *et al.*, 2008; Lopes-Serrao *et al.*, 2011; Thompson *et al.*, 2010).

Over recent years, however, it has become evident that dose-reductions from planned therapy adversely impacts survival from hematologic malignancies (Hunter *et al.*, 2009; Lyman, 2009) and that dosing per actual body weight is likely safe in hematologic malignancies, resulting in no significant differences in TRT (Abdah-Bortnyak *et al.*, 2003; Hourdequin *et al.*, 2013; Miyahara *et al.*, 2013). In 2012, the American Society of Clinical Oncology reviewed the literature and issued a clinical practice guideline specifically recommending against dose limitation in obese patients (Griggs *et al.*, 2012). While the guidelines have been overall adopted, controversy surrounding this issue continues, particularly whether the recommendation is appropriate for disparate tumor types (Miyahara *et al.*, 2013; Sandhu *et al.*, 2014) and unique settings such as stem cell transplant (Berger, 2015). Moreover, even the authors of the guidelines acknowledged that data in extreme obesity is limited, and thus the panel could not make specific recommendations for that growing proportion of the population. While the guidelines resulted in a practice trend for the use of dosing per actual body weight, these caveats precluded their implementation as a blanket recommendation for all obese patients. While the majority of patients can tolerate –and should be dosed– according to actual body weight, cancer type, chemotherapy and potential pre-existing obesity-associated organ dysfunction must all be included in clinical decision making, particularly in older adult populations.

20.2.3 Obesity and survival from adult hematologic malignancies

Due to the wide range of practices for dose delivery of chemotherapy as described above, it is difficult to determine the precise association of obesity and survival; what is clear, however, is that obesity exerts a non-uniform effect on survival across leukemia subtypes and age. The Cancer Prevention Study II was the first study to conclusively connect obesity with survival relative to the general adult population, although this was presumed at the time to be principally due to the development of a malignancy as opposed to interaction with the therapy itself. Likewise, while this was concluded based on examination of broad categories of malignancies such as

'non-Hodgkin lymphoma' and 'leukemia,' the effect of obesity on mortality within the different subtypes remained unresolved. Similarly, a subsequent meta-analysis of leukemia mortality in adults showed increased risk present in obese patients, but was also limited to combining multiple forms of leukemia within a single analysis (Castillo *et al.*, 2012). When the potential interaction of obesity and survival is examined within specific malignancies, once again the trend for a differential effect of obesity by cancer type emerges.

CLL and AML constitute the two most common adult leukemias and yet between them, obesity has a dichotomous effect on survival. In a case control study of CLL, obesity during therapy is associated with a dose-dependent effect on mortality; overweight patients with CLL have a more than two-fold risk of dying from disease as compared to normal weight patients; obese patients have more than double that risk, with a nearly five-fold risk of dying from refractory or relapsed disease (Leo *et al.*, 2014). A meta-analysis of prospective studies further supports the dose-dependent nature of this interaction; every increase in BMI of 5 kg/m^2 was associated with a 14% increase in risk for dying from CLL (Larsson and Wolk, 2011). Conversely, two studies focusing specifically on adult AML showed no effect of obesity at diagnosis on survival. Although the impact of obesity-related empiric dose reductions in AML on TRT and disease response is unclear (Lin *et al.*, 2013), it should be noted that in both of the above studies chemotherapy was dosed as per actual body weight (except for one patient with extreme obesity) (Medeiros *et al.*, 2012; Wenzell *et al.*, 2013). In one study of AML in older patients (≥60 years), obesity was even associated with improved survival (Brunner *et al.*, 2013), while in APL, a unique subtype of AML, obesity was associated with increased relapse (Breccia *et al.*, 2012). In the less common CML, obesity has also been associated with both delayed response to therapy (twice as long to achieve a complete cytogenetic response) and also a 25% reduction in likelihood of ever achieving that complete response (Breccia *et al.*, 2013). Interesting, while ALL is far less common in adults, no data has been published to date examining obesity and survival. This is particularly concerning due to the strong association of ALL and outcomes in pediatric populations described below. In multiple myeloma, a recent meta-analysis similarly found a dose-dependent effect of overweight and obesity on survival (Wallin and Larsson, 2011). Thus, while comparison of exact outcomes remains complicated by the variability in practice for weight-based dosing of chemotherapy, it nonetheless remains clear: (1) obesity's influence cannot be evaluated through such a broad lens as general 'leukemia,' and (2) the distinct biology of the different leukemia itself likely plays a role in obesity's modulation of survival.

20.2.4 Obesity and pediatric leukemia outcomes

Disease prevalence markedly shifts in children, with ALL and AML constituting over 90% of all diagnosed hematologic malignancies (Ward *et al.*, 2014). As discussed above, the growing obesity epidemic in pediatric populations mirrors that of adults. While no studies reported on whether a similar association of obesity and leukemia incidence occurs in children, similar questions arose in clinics regarding the safety of chemotherapy dosing. As opposed to the adult population, however, these questions were addressed systematically via examination of large national clinical trials. The first such study to report on obesity during therapy was a randomized international

clinical trial conducted by the (now named) Children's Oncology Group in pediatric AML (CCG protocol 2961). Midway through the trial, the study chairs began to notice an apparent pattern of greater TRM among the obese. They therefore conducted a formal interim analysis to validate their anecdotal observations, characterize the effect size, and explore potential etiologies of obesity's contribution to TRM. The results of this analysis, published in 2005 in the Journal of the American Medical Association (JAMA) demonstrated for the first time on a large, cooperative group cancer trial a clear effect of obesity affecting leukemia survival despite uniformity of disease and therapy (Lange *et al.*, 2005). The authors found that the effect of survival was due not to refractory disease or relapse, but to TRM alone. Children obese at diagnosis possessed a greater than three-fold risk of dying from a complication during early therapy than those of a normal weight; the principal TRT being severe infection. With the caveat that the study was not designed to examine the etiology of this unanticipated adverse effect, the authors found no evidence of increased chemotherapy toxicity based on hematological criteria. Subsequently, data from trials conducted within the St. Jude Research Consortium confirmed an identical effect of obesity on TRM, again primarily due to infectious toxicity (Inaba *et al.*, 2012). As opposed to adults, obesity in pediatric AML is clearly associated with poorer survival due to TRT and TRM.

As ALL is the most common pediatric malignancy, constituting 25% of all childhood cancers (Ward *et al.*, 2014), attention soon turned to determine whether a corresponding effect of obesity was present in ALL therapy. The Children's Oncology Group again turned to its clinical trials and in a combined study of almost 6,000 patients accrued from six consecutive completed cancer trials (1988-2002) obesity at diagnosis was found to adversely affect survival (Butturini *et al.*, 2007). Contrasting with pediatric AML, no evidence was found for an effect of obesity on TRM or severe TRT in ALL therapy. Instead, obesity was found to be associated specifically with a 50% increased risk for relapse, particularly in the pre-adolescent and adolescent patients older than ten years. In parallel, the St Jude Research Consortium examined four completed clinical trials (1988-2000) but found no effect of obesity at diagnosis on risk for relapse of ALL or survival (Hijiya *et al.*, 2006). A potential reconciliation of this discrepancy might lie in a common limitation shared by both studies. As opposed to AML therapy which consists of several cycles of chemotherapy over a relatively short duration, ALL is treated with months of intensive chemotherapy followed by years of low-intensity 'maintenance' chemotherapy. Weight status in ALL therapy fluctuates significantly during these prolonged therapies based on dose-intensity, regimen, and phase of treatment (Withycombe *et al.*, 2009). Extrapolating a single weight at diagnosis to define obesity for years of therapy therefore is not logical. In fact, a follow-up study in the Children's Oncology Group tested one such implied hypothesis: because weight changes over the course therapy, the effect on disease response/relapse is not fixed at diagnosis, but rather is a cumulative effect of being exposed to obesity during treatment (Orgel *et al.*, 2014a). The authors found that only those children who remained obese for over half of the intensive pre-maintenance therapy experienced greater risk for relapse, those who attained a normal weight during the same period faced no more relapse risk than one never obese. When toxicity was assessed within each cycle in the context of weight, significant differences were found in risk for TRT in those obese vs. normal weight. The obese patients were at specifically greater risk for severe liver and pancreatic toxicity which is consistent with obesity's end-organ effects (Hong *et al.*, 2011; Marchesini *et al.*, 2008).

As a secondary analysis, delivery of target dosing was not available and whether these toxicities affected dose delivery is unknown. Obesity thus adversely affects chemotherapy efficacy and non-fatal toxicity in pediatric ALL.

This longitudinal assessment of obesity during ALL therapy also suggests the exciting possibility that the effect of obesity is not fixed at diagnosis, but that normalization of weight may be able to reverse the effect of obesity on disease response. This would imply that obesity's influence on survival is not via permanently altering the biology of the leukemia cell itself during the leukemogenic event, but rather that obesity modulates disease response through constant exposure. Such an ongoing effect could then potentially be reversed through an obesity-targeted intervention in the host (child). A subsequent institutional report by the same investigators further clarified the timing of onset. Since obtaining a deep remission in the first month of therapy (induction) is crucial to long-term survival (Borowitz *et al.*, 2008), the authors examined end of induction disease response in obese vs. non-obese children newly diagnosed with ALL. They found that obese children were far more likely to have a poorer disease response even this early in therapy, with shallower remissions and increased residual leukemia at the end of induction. Similar to the earlier study of cumulative obesity exposure, evidence for obesity's ability to modulate relapse risk post-induction was present in this study as well (Orgel *et al.*, 2014b). Accordingly, while duration of obesity remains important, this second study clearly highlights the ideal time to intervene begins at diagnosis, despite this being a complex period of psychosocial stressors and physical comorbidities from the new-onset leukemia. Nonetheless, as obesity constitutes one of the few potentially modifiable risk factors for poorer survival in pediatric ALL, and as ALL relapse remains the number one cause of death in pediatric ALL, such interventions promise new approaches to augment the efficacy of our current best therapies.

20.2.5 Obesity's influence on stem cell transplant

One other patient group requiring mention is the unique stem cell transplant population, a treatment modality often restricted to adults and children with severe and/or refractory hematologic malignancies. In this setting, chemotherapy is dosed at extremely high levels not only to preferentially destroy the targeted cancer cell but also to kill the healthy cells in order to ready the bone marrow compartment for stem cell infusion (i.e. myeloablative dosing). However, appropriate dosing for myeloablative chemotherapy in the obese remains an open question. This has particular relevance on transplant outcomes where hepatic synthetic function is of absolute importance for safe delivery of the chemotherapy; the concern in this situation is therefore that any effect of obesity on hepatic function may predispose patients for added transplant toxicity, specifically for fatal hepatic failure due to sinusoidal obstructive syndrome (a chemotherapy-induced vascular congestion in the biliary system) and/or graft-versus-host disease (a condition where the transfused stem cells attack the host and cause organ damage leading to dysfunction or organ failure). Large registry studies in the Center for International Blood and Marrow Transplant Research database showed no effect of obesity on transplant outcomes in either adult AML or pediatric hematologic malignancies (ALL or AML) (Aplenc *et al.*, 2014; Navarro *et al.*, 2010). Nonetheless, limitations inherent to both studies remain the recurrent theme; neither

study could include the unknown empiric dose adjustments for obesity at the individual centers. While several non-rigorous retrospective reviews and small studies report on the issue (Bubalo *et al.*, 2014), the American Society for Blood and Marrow Transplantation could not find sufficient evidence for a formal recommendation on obesity and instead published a structured review for continued individual interpretation (Bubalo *et al.*, 2014). The heightened stakes from pushing to the limit of organ toxicity already present in the transplant settings makes further investigation into dosing practices for obesity imperative to optimize transplant outcomes.

In summary, obesity has a pervasive adverse influence on survival for the majority of hematologic malignancies. A noted exception, and one affecting a large proportion of patients affected by a hematologic malignancy, is in adult AML where obesity does not appear to adversely impact survival. In pediatric leukemia, obesity has an effect on survival albeit through vastly differing mechanisms. These differences imply that the physiologic linkage between obesity and hematologic malignancy differs among populations, with different pathways present by age, tumor type, or even genetic susceptibility.

20.3 The missing link: the plausible biology of obesity and leukemia

20.3.1 Pharmacokinetic and pharmacodynamic considerations

While epidemiologic studies have established the association, the causality and pathophysiology remain unclear. In searching to find a common mechanism across a myriad of adult and pediatric cancers, each with a unique inciting cell and biology, the initial focus centered on the one commonality, the nature of the obese host. In the general population, obese individuals have altered drug clearance due to changes in renal filtration and hepatic conjugation (Hanley *et al.*, 2010). This is further complicated in the treatment of hematologic malignancies due to chemotherapy's effect on albumin/protein synthesis (i.e. a primary determinant of its volume of distribution), variable renal and hepatic function from expected toxicity, and finally, the lipophilic properties of some chemotherapies that may decrease effective clearance of the drug. A recent structured review of pharmacokinetic studies of common chemotherapy agents demonstrated no consistent pattern for volume of distribution and clearance, likely due to this complicated milieu of pharmacokinetics and pharmacodynamics (Hall *et al.*, 2013). If anything, small studies of the drugs most common to therapeutic regimens for hematologic malignancies (e.g. anthracycline, methtotrexate, steroids, cyclophosphamide, cytarabine, etoposide) demonstrated either no effect of obesity or decreased clearance (Dunn *et al.*, 1991; Hall *et al.*, 2013; Rodvold *et al.*, 1988; Thompson *et al.*, 2009; Zuccaro *et al.*, 1991). While one might draw a conclusion for dose reduction in those with decreased clearance, caution must be used in interpreting these small reports, especially in the context of clinical studies showing tolerability for chemotherapy dosed per actual body weight (Gamis *et al.*, 2014; Hijiya *et al.*, 2006; Wenzell *et al.*, 2013). For now, the only conclusion that can be reliably drawn from the available literature is that, similar to the nature of obesity's effect on malignancies, chemotherapy cannot be considered a single category for terms of guidelines, but should be examined individually for the pharmaceutical properties

affecting efficacy; either to empirically dose reduce for toxicity or to allow for pharmacokinetic-mediated dose 'escalation' in the obese to maximize efficacy.

20.3.2 Leukemia biology in the obese

As hematologic malignancies are inherent to the immune system, one must also consider the interaction of obesity on the immune environment of the host. Early studies of leukemia in those with high birth weight (Caughey and Michels, 2009; Hjalgrim *et al.*, 2003), and of adults with diabetes mellitus and insulin resistance (Orgel and Mittelman, 2013), implicate the IGF-1 axis as a leukemia-promoting mechanism. IGF-1 has the ability to potentiate growth of malignancies directly via expression of the IGF-1 receptor on tumor cells as well as via signal transduction pathways implicated in leukemia initiation and progression (Rose and Vona-Davis, 2012). Correlative laboratory models have supported IGF-1's role as a central mediator of leukemogenesis both using *in vitro* studies of IGF-1's mitogenic effect on ALL and AML cell lines (Shimon and Shpilberg, 1995) as well as through leukemia progression seen *in vivo* in 'fatless' mouse models with astronomical levels of IGF-1 but lacking other obesity-associated changes (Hursting *et al.*, 2007). Insulin resistance in the obese, and resultant over-expression of the IGF-1 axis, is one such plausible mechanism connecting obesity to the development of hematologic malignancies.

Obesity does not act on the system only through one pathway, but exhibits a global effect too. Obesity results in a constant overall pro-inflammatory state with increased circulation of pro-inflammatory cytokines and growth factors (IGF-1, IL-1β, IL-6, and TNF-α) (Makki *et al.*, 2013). Obesity also affects circulating levels of adipokines (hormones released from fat cells) with greater concentrations of circulating leptin and inhibited production of adiponectin. The change in adipokines and pro-inflammatory state combine to activate multiple signal transduction pathways (PI3K/AKT/mTOR, JAK/STAT and MAPK/ERK) implicated in leukemogenesis (Chen, 2011; Kim *et al.*, 2010; Turzanski *et al.*, 2004). Moreover, leukemia cells demonstrate a high expression of leptin receptors and changes in adipokines can also directly stimulate proliferation of leukemia cells and inhibit their apoptosis (cell death) (Bruserud *et al.*, 2002; Konopleva *et al.*, 1999; Obeid and Hebbard, 2012). The biological link between obesity and hematologic malignancies may therefore not reside within the host-chemotherapy interaction, but through alteration of intracellular signaling and the biology of the leukemia cells themselves.

20.4 Conclusions

Multiple epidemiologic studies have conclusively established an association with both the incidence of hematologic malignancies and survival from the many subtypes. Ongoing research must now turn to focus on the biology and pathophysiology to answer key questions relevant to public health (i.e. cancer prevention) and therapy outcomes (i.e. treating obese patients). As outlined above, the recurring theme is one quite familiar to obesity investigators; the answers likely lie not within one avenue but are the result of many intersecting physiological systems affected by the pervasive influence of obesity. As such, while the biology must be further characterized, the

solution will also likely be one equally familiar, the urgent need to develop feasible obesity-targeted interventions not only in the general population, but in those undergoing chemotherapy as well.

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21. Nutrition in anemia

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Abstract

Anemia is a condition in which hemoglobin level is two standard deviations below the mean for a particular age or sex. The cause of anemia varies with age and sex. Dietary deficiency of hematinic factors like iron or vitamins like folic acid and vitamin B12 are the most important treatable and preventable causes for anemia. Most susceptible populations are infants, preschool children, pregnant and lactating females. In pregnancy the woman loses iron to provide for the iron stores of the fetus. Besides diet causes, some chronic illnesses like chronic kidney disease, rheumatoid arthritis, etc., can also lead to anemia. Symptoms in anemic patients arise from lack of oxygen in blood and these symptoms are fainting, breathlessness on exertion, tiredness, sleeplessness, pallor, insomnia, dysguesia, etc. Diet has a major role in both prevention and treatment of anemia, iron deficiency or megaloblastic. A dietician should be involved in the initial management both to review the dietary history as well as advising on the practical issues of dietary management. Some practical tips in the dietary management of anemic patients are discussed in this chapter.

Keywords: iron, folic acid, ascorbic acid

Key facts

- School-age children with hemoglobin (Hb) levels lower than 11.5 and 12 g/dl were considered as anemic for age.
- Mild anemia is defined as Hb concentrations between 10-11.9 g/dl for 12-15 years and Hb concentrations of 10-11.4 g/dl for 5-11 years children. Moderate anemia is defined as Hb concentrations between 7-9.9 g/dl and severe anemia defined as Hb concentrations lower than 7 g/dl.
- Anemia is very common in developing countries and the most common type is iron deficiency anemia.
- In iron deficiency anemia serum iron and ferritin levels are below 10 µmol/l and 15 µg/dl, respectively.
- The iron content of breast milk is low and is inadequate for the predominantly breast fed full-term infant after the age of six months.

Summary points

- The WHO guidelines label a patient anemic when the cut-off levels for the lowest value of Hb are: 6 months to 6 years: 11 g/100 ml; 6-12 years: 12 g/100 ml; >12 years male: 13 g/100 ml; >12 years female: 12 g/100 ml; and pregnant ladies: 11 g/100 ml.
- Menstrual blood loss accounts for higher iron requirements in female as compared to their male counterpart. Therefore an increased iron intake should be recommended.
- Average iron content in the human body is 40-50 mg iron/kg body weight.
- Besides increasing Hb content, iron plays an important role in transport of oxygen and in conversion of blood sugar to energy.
- Dark chocolate, sun dried tomatoes, baked potato with skin, chopped and boiled spinach, tofu, prune, raisins, soybean, pinto beans, lentils are some of the richest sources of iron.
- Vitamin C, beef, poultry, salmon are iron absorption enhancers whereas phytic acid, egg protein, tannic acid, coffee, cocoa are iron absorption inhibitors.
- Heme iron is better absorbed directly in the human gut to the extent of 60-70%.
- Various doses of phytate reduced iron absorption by 10 to 50%. But adding 50 mg of vitamin C increased iron absorption to almost 30%.
- Cabbage, lemon, amla, turnip green, drumstick leaves are some of the richest sources of vitamin C.

Abbreviations

FNB	Food and Nutrition Board
Hb	Hemoglobin
RDA	Recommended dietary allowance
RBC	Red blood cell

21.1 Introduction

Anemia is a condition in which blood does not have enough Hb or RBCs (Mohapatra *et al.*, 2014). Blood sustains life. It plays a vital role in supplying oxygen, nutrients, vitamins, minerals, trace elements, etc. to each and every cell of the body. A deficiency in the supply or quality of blood will impair the quality of life.

21.1.1 Definition of anemia

Anemia is defined as Hb levels lower than 11.5 g/dl and 12 g/dl in school-going children of age 5-11 and 12-15 years old, respectively. Mild anemia is defined as a Hb concentration between 10-11.9 g/dl for children between 12-15 years and a Hb concentration of 10-11.4 g/dl for school going children of 5-11 years. Moderate anemia is defined as a Hb concentration between 7-9.9 g/dl and severe anemia is defined as a Hb concentration lower than 7 g/dl. Iron deficiency anemia is a condition in which serum iron levels are below 10 μmol/l and ferritin levels are below 15 μg/dl (Lee and Herbert, 1999; Osazuwa and Ehigie, 2010).

21.1.2 Causes of anemia

Among the various factors that lead to anemia the most important treatable and preventable causes are deficiency of iron or vitamins like folic acid and vitamin B12. An anemic patient will have either: (1) decreased RBC compared to normal; or (2) decreased Hb level. Hemoglobin is a substance containing iron (heme) and protein (globin).

Besides nutritional deficiencies, anemia can result from a variety of causes of which some important causes are:

- Menstrual blood loss in females belonging to reproductive age group.
- Bleeding from peptic ulcer or due to any other cause be it internal bleed or external visible blood loss.
- Besides dietary deficiency, imbalance between demand and supply is another important cause. Increased need of iron which when not fulfilled can lead to anemia like in pregnancy, lactation, growth spurts, etc.
- Chronic illnesses like chronic kidney disease.

21.1.3 Iron requirements throughout the human lifecycle

Iron is present in most of our daily food items but the quantitative availability varies and hence they are termed iron-rich food or low-iron foods. Dietary iron intake is usually related to energy intake. Our body needs iron to make Hb. It is an essential element for almost all living organisms. Iron requirements are highest in the second and third trimesters of pregnancy, infancy, during growth spurts, etc. Requirements are also high in young children, particularly between 6-18 months of age. Once birth iron reserves are exhausted, infants depend on weaning foods for iron as the iron content of human milk is low. Requirements increase during adolescent growth spurts and by the onset of menstruation in girls. Menstruation increases the average daily iron loss to about 2 mg per day in premenopausal female adults.

Normal value of Hb is 15 g per dl (100 ml). WHO has given levels of Hb for different categories (WHO, 2001). Any individual with a lower level is labeled as anemic (Table 21.1).

Iron requirements are particularly high at certain stages of life, and therefore less likely to be met in those periods causing an iron deficiency state. Iron requirements are highest for pregnant ladies: 1.9 mg/1000 kcal of dietary energy in the second trimester and 2.7 mg/1000 kcal in the third trimester. The figures for other groups are: infants 1.0 mg/1000 kcal; adolescent boys 0.6 mg/1000 kcal; non pregnant women 0.6 mg/1000 kcal; preschool and school children 0.4 mg/1000 kcal and adult men 0.3 mg/1000 kcal. Menstrual loss puts females at greater risk than men as there is an extra requirement to replace iron losses in menstruation. Hence, most women enter pregnancy with depleted iron stores and suffer from anemia. Besides there is also a need to increase iron content in the diet of a pregnant female so as to fulfill the need of iron by a developing fetus and to compensate the blood losses at the time of delivery. In infancy, risk factors for anemia include premature clamping of the umbilical cord and maternal iron deficiency during pregnancy. The iron content of breastmilk is low and is not enough for infant after six months. Therefore, it is always advisable to introduce weaning foods to the infants after six months of age.

Table 21.1. The cut-off levels of hemoglobin in different age groups.

Age	Hemoglobin (g/100 ml)
6 months to 6 years	11
6-12 years	12
>12 years Male	13
>12 years Female	12
Pregnant ladies	11

21.1.4 Common symptoms of anemia

Anemic patients are symptomatic due to lack of oxygen in blood. These symptoms can be enumerated as:

- weakness;
- fainting;
- breathlessness on exertion;
- tiredness and lethargy;
- sleeplessness;
- insomnia;
- change in taste or loss of appetite.

21.2 Diet and anemia

Diet plays a major role in anemia prevention and treatment. A dietician should be involved in the initial management of anemic patients both to review the dietary history as well as to advise on the dietary management. Some practical tips in the management of anemic patients will be described in the following paragraphs.

21.2.1 Iron intake

Diet comes to layman as food providing carbohydrates, protein and fats. However the roles of vitamins and minerals cannot be underestimated. Hence we should be aware of sources of important vitamins and minerals, in particularly iron, as its deficiency is one of the major causes of anemia. Iron is present in beef, chicken, liver, pork, turkey, fish, prunes, raisins, apricots, spinach, beetroot, carrot, lentils and beans, etc.

21.2.2 Iron in meat vs. plants

Iron is prevalent in wide variety of plant foods (Table 21.2, Figure 21.1 and 21.2), especially lentils and beans, dark green leafy vegetables and grains. This is important to us as bulk of our diet comprises of vegetables or pulses. This is more pronounced in developing countries like India where nonvegetarian diet is a costly affair. Hence the following plant sources with good iron content can be recommended to vegetarians with iron deficiency.

The primary source of dietary heme iron is red meat. In meat, 40% of iron is bound to the heme molecule as Hb and myoglobin (Anderson and Fitzgerald, 2010). Heme iron has best absorption as compared to non heme iron. 100 g edible portion of beef contains about 18.8 mg iron while beef muscle contains 0.8 mg; mutton muscle contains 2.5 mg iron. The rest of the iron in meat and in all iron plants is non-heme iron. Non-heme iron requires being released from food components by hydrochloric acid and the digestive enzyme pepsin in the stomach (Groff and Gropper, 2000). Next, transferrin is required for transport of iron from the absorption site to the storage site.

Table 21.2. Iron content of some plant foods.

Foods	Preparation	Serving	Iron (mg)
Dark chocolate		100 g	6.3
Broccoli	chopped, boiled	½ cup	0.52
Peas	boiled	½ cup	1.2
Spinach	chopped, boiled	±115 g	3.2
Kale	chopped, boiled	½ cup	0.59
Sweet potato	baked, with skin	35-40 g	0.7
Potatoes	baked, with skin	1 medium size	3.2
Tomatoes	cooked	115 g	0.82
Tomatoes	sun dried	1 cup	4.9
Strawberries		1 pint	1.5
Oatmeal	cooked	115 g	1
Whole wheat pasta	dry	¼ cup	0.4
Rice (white, long-grain, unenriched)	cooked	½ cup	0.7
Rice (white, long-grain, enriched)	cooked	½ cup	1.4
Brown rice	cooked	1 cup	0.8
Bread-whole wheat		1 slice	0.68
Tofu		½ cup	3.4
Soymilk		1 cup (±225 ml)	1.0-1.5
Prune	juice	1 cup	3.0
Pinto beans	boiled	1 cup	3.6
Kidney beans	boiled	½ cup	2.6
Soybean	boiled	1 cup	8.8
Peas-green	boiled	½ cup	1.2
Lentils	boiled	1 cup	6.6
Brussel sprouts		½ cup	0.9
Cashewnut	dry roasted	50 g	2.90
Peanut butter		2 tablespoons	0.6
Sesame butter		1 tablespoon	0.4
Walnuts	chopped	¼ cup	0.85
Almonds	roasted	¼ cup	1.3
Pistachios	dry roasted	25 g	1.2
Sunflower seeds	dry roasted	25 g	1.2
Pumpkin seeds	dry roasted	1 ounce (about a handful)	0.9
Dried figs	dried, raw	½ cup	1.5
Peaches	dried	¼ cup	1.6
Raisins		25 g	1.4
Apricots	dried	½ cup	2.0
Grape nuts		½ cup	16
Total, whole grain		±115 g	8
Black beans	boiled	1 cup	3.6
Black eyed peas	boiled	1 cup	4.3
Molasses		2 tablespoons	3.8
Thyme	dried	1 teaspoon	1.2

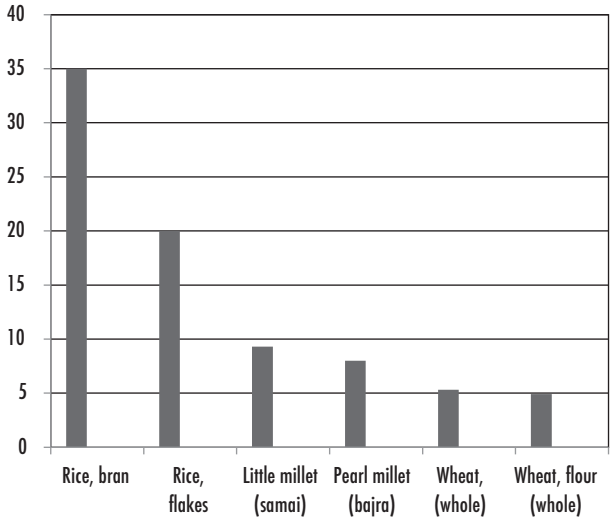

Figure 21.1. Iron content (mg/100 g edible portion) of cereal grains and their products.

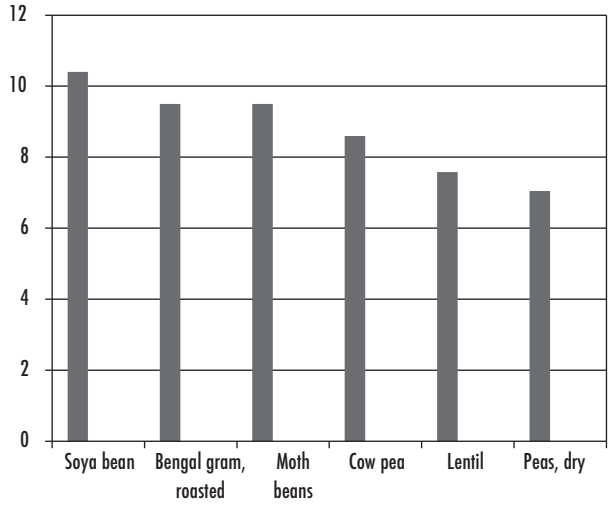

Figure 21.2. Iron content (mg/100 g edible portion) in pulses and legumes.

21.2.3 Iron absorption enhancers

In order to increase iron absorption from a diet containing non-heme iron we can use some iron enhancers. Some of the iron absorption enhancers are:

- beef;
- fermented vegetables;

- fermented soy sauce;
- poultry (Hurrell *et al.*, 2006);
- salmon (and presumably, some other kinds of fish) (Navas-Carretero *et al.*, 2008);
- pork (Engle-stone *et al.*, 2005);
- citric acid (Hallberg and Rossander, 1984);
- vitamin C or ascorbic acid (Fidler *et al.*, 2009; Teucher *et al.*, 2004) will overcome the negative effect on iron absorption of all inhibitors like polyphenols, phytates and the calcium and proteins in milk products. Vitamin C is the best enhancer in vegetarian diet.

When talking about iron absorption enhancers we should be aware of the inhibitors as well.

21.2.3 Iron absorption inhibitors

Substances that inhibit absorption of iron are called iron absorption inhibitors which are enumerated as given below:

- phytic acid and other inositol phosphates (found in grains, legumes and other plant foods) (Kishore, 2006), are the main iron absorption inhibitors;
- animal proteins such as milk proteins and egg protein (Hurrell *et al.*, 1988; Hurrell *et al.*, 1999);
- soybean protein also decreases iron absorption (Lynch *et al.*, 1994);
- minerals that compete with iron for absorption for example calcium, zinc, magnesium, and copper;
- tannic acid found in tea (Kishore, 2006);
- coffee (NutritionMD, undated);
- cocoa (NutritionMD, undated).

Many nutritious foods do contain iron absorption inhibitors. These food items do provide calcium, zinc, phosphorous and fiber, hence cannot be excluded totally from our usual diet.

21.2.5 Factors affecting absorption of iron present in foods

Heme and non-heme iron

As discussed previously iron in our diet is usually present in two forms: heme and non-heme iron. Heme iron is present mainly in Hb and myoglobin. Heme iron derived from animal food is easily absorbed in the human body: you eat it and you absorb it (Table 21.3). Food composition and gastrointestinal secretion affect the heme iron absorption. All non-meat based iron i.e. plant sources such as lentils and beans, iron fortified cereals, green leafy vegetables, pulses, etc. are the main source of non-heme iron (Table 21.4). The absorption of non-heme iron is increased by the presence of ascorbic acid. Human beings usually obtain 60-65% of our iron requirements from non-heme iron.

Table 21.3. Heme iron foods.

Food	Iron content (mg)
100 g mutton muscle	2.5
100 g rohu	1.0
90 g lamb liver	8.6
100 g prawn	5.3
100 g lean beef	2.2
100 g pork, muscle	2.2
120 g fish (non specified type)	1.1
100 g chicken breast	0.7

Table 21.4. Non-heme iron foods.

Food	Iron content
30 g bran flakes	5.4
1 cup cooked beet greens	2.7
1 cup cooked turnip greens	3.2
½ cup canned chickpeas	1.6
6 ounces soy yoghurt	1.1
½ cup spinach	0.6
1 large potato	3.2
1/8 medium watermelon	1.4

Role of the stomach

Since iron is absorbed in the ionic state, it is judicious to suppose that gastric digestion may help in solubilizing dietary iron, hence iron absorption is impaired in hypoacidity. The assimilation of iron may be impaired by rapid emptying of the food from the stomach. It has been demonstrated that much more iron can be extracted from food materials by acid peptic digestion than by saline extraction (Swaminathan, 2003a).

Ascorbic acid

Vitamin C or ascorbic acid helps in iron absorption even in presence of iron inhibitors. Vitamin C is the only dietary constituent other than animal tissue that has been shown repeatedly to

augment the absorption of non heme iron in humans. (Ballot *et al.*, 1987; Callender *et al.*, 1970; Cook and Monsen, 1977; Hallberg *et al.*, 1986; Moore and Dubach, 1951).

Vitamin C is a strong enhancer of plant iron and can overcome the inhibitors in plant foods. One study found that various doses of phytate reduced iron absorption by 10 to 50%. But adding 50 mg of vitamin C increased iron absorption to almost 30%. Similarly, in the presence of a large dose of tannic acid, 100 mg of vitamin C increased iron absorption from 2 to 8% (Hallberg *et al.*, 1991).

Hence it is advisable to consume iron along with vitamin C, as this will result in a more rapid absorption of iron. Besides, vitamin C plays a vital role in the synthesis of RBCs as well as the integrity of blood vessels (Teucher, 2004). In Table 21.5 several dietary sources of vitamin C can be found.

Phytic acid

Phytates are found in grains, seeds, nuts, vegetables, fruits, etc. Chemically, phytates are the hexaphosphoric acids of inositol and are a storage form of phosphate and minerals. Brans of cereals have a high content of phytates and thus they inhibit iron absorption. In bread, during

Table 21.5. Dietary sources of vitamin C.

Food, standard amount	Vitamin C (mg)	Calories (kcal)
Redgram. tender	25	116
Cabbage	124	27
Carrot leaves	79	77
Coriander leaves	135	44
Drumstick leaves	220	92
Fenugreek leaves	52	49
Turnip green	180	67
Sweet potato	24	120
Turnip	43	29
Bitter gourd	88	25
Capsicum	137	10
Amla	600	58
Cape gooseberry	49	53
Lime	63	59
Strawberry	52	44
Coconut, dry	7	662
Chillies, dry	50	246
Chillies, green	111	29

fermentation of dough, some of the phytic acids in bran are degraded. The process of fermentation degrades the phytate and increases the bioavailability of iron in whole wheat bread.

Polyphenols, which include tannic acid, can inhibit iron absorption, and are found in coffee, cocoa, and black, green and many herbal teas. You should avoid these foods at meals if you are trying to increase iron absorption (Hurrell, 1999).

Oxalic acid

Oxalic acid present in certain vegetables forms insoluble iron oxalate and prevents the absorption of dietary iron (Swaminathan, 2003a).

Tea and coffee

In developing countries the cause of iron deficiency is inadequate iron but in developed countries the cause of iron deficiency is consumption of tea or coffee particularly after meals. In individuals with low intakes of heme iron, low intakes of enhancing factors and/or high intakes of inhibitors, iron absorption may be an issue. For subjects at risk of iron deficiency, the following recommendations are made: (1) increase intake of heme iron (this form of dietary iron is present in meat, fish and poultry and is hardly influenced by other dietary factors with respect to its absorption); (2) increase meal time ascorbic acid intake; and (3) fortify foods with iron. Recommendations with respect to tea consumption (when in a critical group) include: (1) consume tea between meals instead of during the meal; and (2) simultaneously consume ascorbic acid and/or meat, fish and poultry (Zijp, 2000).

Effect of other dietary factors on iron absorption

The amino acid, L-lysine, plays a part in the absorption of iron and zinc. Among plant foods, L-lysine is found in high amounts mainly in legumes (peanuts, beans, lentils, and peas) and vegans that do not eat many legumes could find themselves falling short on lysine. In some women, iron supplements do not lead to an increase in iron stores. But according to one study of such women, adding the amino acid L-lysine (1.5-2 g/d for six months) to iron supplementation did increase iron stores (Rushton, 2002).

21.3 The food and nutrition board and vegetarian diets

The FNB of the Institute of Medicine (Institute of Medicine, 2001) set the RDA and DRI for nutrients (Table 21.6 and 21.7). The FNB suggests that iron in vegetarian diets is absorbed at the rate of 10% compared to 18% from omnivorous diets and that iron absorption could be as low as 5% for vegans. The FNB does not explicitly give a separate RDA, but says that the requirement for iron 1.8 times higher for vegetarians (Norris, 2013).

Table 21.6. Recommended Dietary Allowances for iron by age and sex.

Group	Age	Iron (mg/d)
Infants	0-6 months	0.27[1]
	7-12 months	11
Children	1-3 years	7
	4-8 years	10
Males	9-13 years	8
	14-18 years	11
	19-30 years	8
	31-50 years	8
	51-70 years	8
	>70 years	8
Females	9-13 years	8
	14-18 years	15
	19-30 years	18
	31-50 years	18
	51-70 years	8
	>70 years	8
Pregnant women	14-18 years	27
	19-30 years	27
	31-50 years	27
Lactating women	14-18 years	10
	19-30 years	9
	31-50 years	9

[1] This value is an Adequate Intake (AI) value. AI is used when there is not enough information known to set a Recommended Dietary Allowances (Institute of Medicine, 2001).

21.4 Dietary instructions to overcome iron deficiency

A healthy diet plays a very important role for a healthy body. We should eat a balanced diet that includes good sources of iron. A healthful diet includes fruits, vegetables, whole grains, fat free or nonfat milk and milk products, lean meat, fish, dry beans, eggs, nuts, and is low in saturated fat, trans fats, cholesterol, salt and added sugars. Avoid drinking tea with meals as this can reduce the amount of iron that is absorbed.

Do not overcook or overheat the food as this results in loss of greater amount of nutrients. In addition to a healthful diet that includes good sources of iron, you can also eat foods that help your body absorb iron better. For example, you can eat fruits or vegetables that are a good source of vitamin C with a food or meal that contains non heme iron. Vitamin C helps your body

Table 21.7. Dietary Reference Intake (DRI) for iron by age and sex.

Age	DRI (mg)	Upper limits (mg)
0-6 months	27	40
7-12 months	11	40
1-3 years	7	40
4-8 years	10	40
9-13 years	8	40
Boys, 14-18 years	11	45
Girls, 14-18 years	15	45
Men, >19 years	8	45
Women, 19-50 years	18	45
Women, >50 years	8	45
Lactating women, 14-30 years	10	45
Lactating women, >30 years	9	45
Pregnant women	27	45

absorb the non-heme iron foods you eat, especially when the food containing non-heme iron and vitamin C food are eaten at the same meal the following recommendations are for specific groups who are at greater risk for iron deficiency.

21.4.1 Babies

- If possible, breastfeed your baby for at least 12 months. Introduce mother's milk to infant as soon as possible after birth. Give them colostrum as it contains antibodies to protect the newborn from infections and at 4-6 months of age, give your baby plain, iron-fortified infant cereal and/or pureed meat. Just two or more servings a day can meet a baby's iron needs at this age. Meals should be prepared at home as far as possible to avoid infections.
- When your baby is about 6 months of age, introduce weaning food to them and include at least 1-2 feedings per day of foods rich in vitamin C with foods that are rich in non heme iron to improve iron absorption.
- Prefer mixed vegetable water (beetroot, spinach, carrot, etc.) or banana as the very first weaning food for your infant as they will provide a good quality iron.
- If you can't breastfeed, use iron-fortified formulas which are easily available. Do consult your doctor or a dietician as they will help you to know the best fortified formula for your baby.
- Don't give low iron milk (e.g. cow's milk, goat's milk and soy milk) until your baby is at least 12 months old.
- If your baby had a premature birth or is born small in size or weight, talk to your doctor about giving iron drops to your baby or if your baby can't get two or more servings per day of iron rich foods (such as iron-fortified cereal or pureed meats) due to any reason, talk to your doctor about giving iron drops to your baby.

In summary, keep an infant on exclusive breastfeeding for the first six months of age. Introduce weaning food along with breastfeeding after six months. Breastfeeding should be continued only up to 2-3 years of age, after that a child should be given a proper diet that contains enough nutrients required for proper growth and development.

21.4.2 Young children

- After your child is one year old, give no more than three 8-ounce servings of whole cow, goat, or soy milk per day. After your child is two years old, low fat or non-fat milk should be used instead of whole milk. Vitamin D-fortified milk is a good source of calcium and vitamin D, but not of iron.
- Always check if your child has any faulty eating habits. Eating of coal can lead to anemia.
- Give your child a diet with iron-rich foods such as iron-fortified bread and iron-fortified cereals and rice and lean meats.
- Include fruits, vegetables or juices that are rich in vitamin C. Vitamin C helps your child absorb non-heme iron especially when the food that is a source of non-heme iron and the vitamin C-rich food are eaten at the same meal.

21.4.3 Adolescent girls and women of childbearing age and pregnant ladies

- Eat iron-rich foods.
- Eat foods that are sources of vitamin C at the same meal because vitamin C helps your body absorb non heme iron especially when the food that is a source of non heme iron.
- Eat red meat, poultry, and fish because iron in animal foods is easier for your body to absorb than the iron in plant foods. Take a balanced diet to overcome any deficiency.

21.5 Folic acid intake

Folic acid is a vitamin needed by the body to make and maintain new cells. It is particularly important for pregnant ladies as it encourages growth of fetus. Besides it is needed for the conversion of homocysteine to methionine (Berg, 1999).

21.5.1 Causes of folic acid deficiency

Causes of folic acid deficiency are:
- poor eating habits;
- poor absorption;
- increased requirements, e.g. due to growth or pregnancy;
- alcoholism;
- oral contraceptives pills.

21.5.2 Foods rich in folic acid

In Table 21.8 foods rich in folic acid are given.

21.5.3 Therapeutic uses and toxicity

Folic acid is administered orally in doses of 5-20 mg daily for the treatment of megaloblastic anemia. It can be given intramuscularly in doses of 2 mg particularly in patients with poor oral absorption. Poor solubility of folic acid increases the chances of crystallization when given in large doses by the parenteral route (Swaminathan, 2003b).

21.5.4 Important guidelines in managing patients with folic acid deficiency

- Include foods rich in folic acid.
- Fruits and vegetables should be eaten raw whenever possible as cooking destroys folic acid.
- Pregnant women have higher requirements for folic acid (1 mg daily) and should take folic acid supplements advised by the doctor/dietician/nurse.
- Increase the use of wheat flour and soya flour in baking and food preparation.

21.6 Vitamin B12 intake

Vitamin B12 deficiency leads to megaloblastic anemia. Megaloblastic anemia is a distinct type of anemia characterized by macrocytic red cells and erythroid precursors that show nuclear dysmaturity. Vitamin B12 is present in meat and products, fish, meat, milk and other dairy products, eggs, fortified breakfast cereals, etc.

Table 21.8. Foods rich in folic acid.

Meat	Legumes	Starches	Fruits and vegetables
Liver (best source)	Dried beans	Wholegrain breads	Spinach
Chicken	Lentils	Wheat flour	Beetroot
Kidney	Spilt peas (dhals)	Potato	Brussel sprouts
Egg yolk	Soy products	Sweet potato	Broccoli
	Almonds		Cabbage
	Nuts		Asparagus
			Banana
			Oranges
			Peaches

Vitamin B12 deficiency can be due to:

- a vegan diet (eating a diet that contains no meat or animal products);
- poor absorption due to certain disease condition;
- surgery to the stomach or intestine;
- oral contraceptives pills.

Nutritional deficiencies are far more common in vegans and those consuming only goat milk. Anemia in infancy may be related to inadequate body stores due to maternal vitamin B12 deficiency and/or prolonged breast milk, which is a poor source of vitamin B12. *Helicobacter pylori* infections are implicated amongst adults. A careful dietary history is essential to rule out a diagnosis of megaloblastic anemia. Peripheral smears in megaloblastic anemia show macrocytosis and hypersegmented neutrophils. Treatment comprises of daily intramuscular vitamin B12 0.5-1 mg for two weeks along with folate supplementation followed by maintained folic acid and vitamin B12 supplementation for at least six months. Dietary suggestions include milk for vegans and fish and meat for non-vegetarians.

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