

Early Nutrition: Impact on Short- and Long-Term Health

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Early Nutrition: Impact on Short- and Long-Term Health

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Preface

Over the past decade, major advances have occurred in our understanding of the interaction of genetics and environment (and particularly diet) in health and disease. Thus, it is very germane now to have a workshop dedicated to a review of nutritional practices and feeding behaviors in infancy and early childhood since these not only have a significant influence on the immediate growth and health of the child, but potentially affect long-term health issues such as obesity and hypertension, which often have antecedents early in life. This volume presents the proceedings of the 68th Nestlé Nutrition Workshop held in October 2010 in Washington, DC. The chapters in this book are organized, like the workshop, in a progression from the newborn period to childhood. They are the work of a selected group of international experts in infant and childhood nutrition, and represent the latest knowledge regarding feeding practices during this time and how those impact growth, development and immediate and long-term health.

A significant body of research has demonstrated a major impact of maternal nutrition during fetal life on later health and development of the newborn. This formed the basis of a robust discussion in the first session of the symposium, and draws our attention to the potential long-term effects of specific macronutrients and micronutrients in the maternal diet during human fetal development. One example of this is the recent data on the impact of dietary lipid components as well as proteins in the maternal diet during fetal and early newborn life on various functional outcomes in the developing infant and child. These data are now informing our feeding practices in the newborn critical care unit, particularly with regard to the use of human donor milk and the use of probiotics in the diet of premature and ill newborns. The use of probiotics in this context still poses challenges, since we can clearly influence the gut microbiome to some extent, but the specific microorganisms that should be used, the dose and administration schedule remain subject to significant debate. Thus, this area of fetal and newborn nutrition and its influence on later health is a vibrant and active subject of discussion and investigation, and holds promise for important discoveries on the role of early

nutritional interventions in both short- and long-term health and development.

It is clear from the results of recent surveys presented in the second session of the symposium that in both the developed as well the developing world, even in countries with vibrant economies, a significant number of infants and young children are not consuming the types of foods that have been recommended to support optimal health. As a consequence, an insufficient intake of selected micronutrients, such as iron and zinc, is highly prevalent, particularly in the developing world. A unique and very successful approach using coated micronutrients for supplementing the diets of vulnerable children to address this serious threat to both immediate and long-term health and development is described in this section of the book. Taste perception plays an extremely important role in food preferences. With the current emphasis on reducing the intake of salt and sugars in the diet, the discussion of the science of taste perception and in particular how it develops during gestation, infancy and early childhood contributed significantly to the overall dialogue on nutritional support during this period. In addition to availability, affordability, taste and cultural preferences, the increasing prevalence of allergic reactions to foods during this time of life often determine the types of foods offered. The discussion of the basis and evolution of immunologic reactions to foods in early life was highly informative to the overall dialogue, and provided the basis for further discussion in the next session which focused on the consequences of weaning and subsequent feeding practices on health during late adolescence and adulthood.

The final session of the workshop spanned diverse areas, and major updates were provided on topics that have seen exciting developments in the recent years. Participants could appreciate new – and in some ways revolutionary – information on the influence of early feeding practices on the later development of a number of health-related issues such as food allergies, later food preferences and eating habits, obesity, bone development, the risk of developing celiac disease in genetically predisposed children, and even – albeit at the moment mostly from studies in animals – on longevity! The microbiome and its influence on growth, weight gain and immediate and long-term health are an emerging area of biology that led to a particularly lively discussion. As always, the discussion provided by those who attended the meeting proved exceptionally interesting and informative.

As the Chairs of this workshop, we are particularly indebted to Prof. Ferdinand Haschke and his colleagues at the Nestlé Nutrition Institute. They provided a format and setting that proved to be perfect for engagement, discussion and learning. On behalf of all those who participated in this workshop, we thank you.

*Hans van Goudoever
Stefano Guandalini
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Foreword

The 68th Nestlé Nutrition Institute Workshop was dedicated to a research area that is gathering a significant amount of interest in the scientific arena: Early nutrition and its impact on long-term health. The International Society for Developmental Origins of Health and Disease has only recently released a position paper on the importance of nutrition in the first 1,000 days [World Nutr 2011;2:195–205].

The scientific evidence from animal and human data showing effects of early nutrition on later health were presented by a group of renowned experts in the field and discussed by an international audience of outstanding health professionals. The topics covered ranged from the nutrition of preterm infants including suggestions on how to improve their short-term outcome by not comprising long-term health, to an evaluation of the current feeding habits of toddlers in different parts of the world. Both problematics, that of ‘over’-feeding and ‘under’-feeding and related malnutrition, were discussed with respect to their long-term outcome. Both weigh equally in their public health burden.

This excellent scientific program was brought together by the three chairpersons, Prof. Hans van Goudoever, Prof. Stefano Guandalini and Prof. Ron Kleinman, to whom we address our special thanks. All three are highly respected in the field of nutrition.

Special thanks go also to Linda Hsieh and her team from the US for her excellent organization and hosting in Washington.

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Animal Studies of the Effects of Early Nutrition on Long-Term Health

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Abstract

Small size at birth is associated with increased risk of a variety of common chronic diseases in adulthood. Numerous experimental studies in animals have supported the observations in humans, demonstrating that changes in nutrition in early life can lead to altered long-term health. Importantly, these effects can be independent of size at birth, and can depend on the interaction between nutritional events before and after birth. Both macro- and micronutrient intake are important. Furthermore, these effects may vary according to the nature, timing, severity and duration of the nutritional insult. This review provides examples from animal studies of evidence of these long-term effects, and some possible underlying mechanisms whereby nutrition in early life can affect long-term health.

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Nutrition and Intrauterine Growth Restriction

Epidemiological studies over the last 20 years have demonstrated that small size at birth is associated with increased risk of a variety of common chronic diseases in adulthood, such as hypertension, coronary heart disease, type 2 diabetes and obesity [1]. This increased risk is not confined to those born with intrauterine growth restriction (IUGR), but appears to be continuous across the birthweight spectrum, including those within the normal birthweight range. The epidemiological evidence from human studies reported by Barker and colleagues, although initially criticized, was rapidly supported by similar observations in animals. For example, the inverse relationship between birth size and adult blood pressure reported in guinea pigs [2] is unlikely to be explained by confounding due to socioeconomic and lifestyle factors.

Previous classic studies in pigs and guinea pigs [3] had demonstrated that undernutrition in early life can result in growth impairment that is permanent, even if nutritional status subsequently improves. Barker thus hypothesized that undernutrition before birth may be a key mechanism leading both to small size at birth and also to the associated long-term outcomes [1].

Animal studies have also demonstrated the important distinction between maternal nutrition, fetal nutrition, and fetal growth. The fetus grows at the end of a long supply line, comprising not only maternal nutrition, but also uterine and umbilical blood flows, placental transport, and placental and fetal metabolism [4]. Therefore, changes in maternal nutrition do not always result in altered fetal nutrition, as there may be sufficient reserve in the fetal supply line. However, as will be discussed below, changes in maternal nutrition can affect long-term health independent of size at birth. Similarly, interference with steps along this fetal supply line in both human and animal pregnancy commonly results in IUGR, since fetal nutrition is the key regulator of fetal growth [4]. These changes also often result in altered long-term health.

The focus of this review is on animal studies that have examined the ways in which nutrition before and after birth can affect long-term health. These effects may vary depending on the nature, timing, severity and duration of the nutritional insult.

Nutrient Balance

Macronutrients

Dietary protein intake varies widely in different communities, and was an early focus for animal studies. Maternal protein restriction during pregnancy in rats resulted in hypertension in the offspring, altered cardiac structure and function, impaired glucose tolerance, and altered fat distribution and food intake [5, 6].

Importantly, postnatal diet, particularly if high in fat, magnified the effects of maternal protein restriction on the offspring. When fed a highly palatable diet from weaning, adult offspring from rat dams exposed to protein undernutrition during gestation and lactation were more insulin resistant, hyperlipidemic, hypertensive, and more obese and sedentary [7]. In utero protein restriction followed by overfeeding during lactation also shortened offspring life span in mice [8].

Other changes in dietary macronutrient balance during pregnancy also alter disease risk in the offspring. Exposure of rat dams to a high-fat diet during gestation resulted in offspring with impaired glucose tolerance, impaired endothelial function, and hypertension [9]. Cardiovascular dysfunction is induced in the offspring by high-fat feeding not only during pregnancy, but

also during lactation. However, if the offspring of dams fed a high-fat diet were also fed a high-fat diet after weaning, the endothelial dysfunction, although not the hypertension, was prevented [10]. In addition, the specific type of dietary fat also modifies the effect of a maternal low-protein diet on offspring blood pressure [11], suggesting that quite subtle changes in the type and balance of dietary macronutrients in early life may have important long-term consequences.

Micronutrients

There are many examples of specific micronutrients in the maternal diet that influence physiology of the offspring. In rats, maternal dietary deficiency of iron, calcium, or zinc during pregnancy is associated with increased blood pressure in the adult offspring [12].

Specific amino acids appear to have particularly important roles in fetal development and subsequent disease risk. For example, taurine is critical in pancreatic and neurological development. Fetuses of rat dams exposed to a low-protein diet had smaller pancreatic islets with reduced rates of islet β -cell proliferation and higher rates of apoptosis, but these effects were prevented by maternal supplementation solely with taurine [13]. In sheep, periconceptual undernutrition resulted in elevated maternal and fetal plasma taurine concentrations in late gestation and evidence of accelerated pancreatic maturation [14]; these changes were associated with later impairment of glucose tolerance in the adult offspring [15].

Similarly, glycine is critical for many aspects of fetal development in utero, including synthesis of DNA, heme, collagen and creatine. In rats, maternal glycine supplementation normalizes the high blood pressure induced in offspring by a low-protein diet in pregnancy, perhaps in part by normalizing endothelial function [16].

Timing and Duration of Undernutrition

Nutritional composition in early life has important effects on long-term function, but the timing and duration of nutritional changes is also important. Maternal undernutrition even before conception in rats resulted in increased blood glucose and cholesterol concentrations in adult offspring [17]. Elevated blood pressure in offspring exposed to a maternal low-protein diet was greater when the dietary insult was initiated very early in gestation than when it was initiated in mid-gestation [18]. Indeed, a low-protein diet in rats solely during the blastocyst stage (first 4.5 days of the 21-day pregnancy) led to postnatal hypertension in the offspring [19].

In sheep, periconceptual maternal undernutrition has been shown to alter many aspects of fetal development in late gestation, including altering fetal growth trajectory [20]. Furthermore, the timing of the periconceptual nutri-

tional insult affects fetal growth and metabolic responses to a late gestation stressor, such as an acute maternal fast [20]. Maternal undernutrition either solely before conception or both before and after conception led to decreased fetal growth in response to an acute maternal fast in late gestation compared with fetuses of ewes well nourished throughout. In contrast, fetuses of ewes undernourished only after conception had no reduction in their growth trajectory in response to the maternal fast [20]. Changes in the fetal glucose-insulin axis or the fetal or maternal hypothalamo-pituitary-adrenal (HPA) axis at the time of the fast cannot explain these differences [12]. Therefore, it seems that preconceptional maternal nutrition has an important role in determining the fetal responses to stress in late gestation [12, 20]. Furthermore, maternal undernutrition can affect fetal development without necessarily limiting substrate supply for tissue accretion, since nutrient requirements in early pregnancy are minimal, and also without exposing the fetus to excess glucocorticoids, since maternal glucocorticoid concentrations were actually decreased during undernutrition [21].

Several aspects of endocrine regulation in late-gestation fetal sheep were also altered by periconceptional undernutrition [4, 12]. Maturation of the glucose-insulin and HPA axes were accelerated [22]. Importantly, these changes persisted after birth, with offspring of ewes undernourished in the periconceptional period showing impaired glucose tolerance which worsened with increasing age [15]. Sheep exposed to periconceptional undernutrition also displayed altered behavioral laterality [23] and suppressed behavioral and glucocorticoid responses to 5 min of isolation, a potent psychological stressor in sheep [24]. These data suggest that maternal undernutrition in early gestation has long-term effects on neurological and endocrine function, and that the effects are demonstrable not only by detailed physiological testing, but also during exposure to the kinds of challenges that might be faced in everyday life.

Undernutrition beyond the periconceptional period also has effects on the health of the offspring. Undernutrition in both early and mid-gestation in sheep led to greater adipose tissue deposition [25], and deleterious effects on the ovaries in fetal and adult life [12] such as reduction in the number of large corpora lutea [26]. In rats, maternal undernutrition in the last third of pregnancy resulted in impaired glucose tolerance in the offspring, whereas a similar period of undernutrition earlier in pregnancy did not [27]. Undernutrition in late, but not early, gestation led to similar effects in ovine studies [28]. Also in sheep, a short, acute maternal undernutrition insult in late gestation increased central HPA axis responses to corticotropic stimulation in adult offspring, independent of birthweight [29]. Interestingly, if the undernutrition insult was long enough to reduce birthweight, the effects on HPA axis function were mitigated. Clearly, maternal undernutrition can result in long-term changes in postnatal physiology without necessarily affecting size at birth.

Possible Mechanisms

Altered Organ Structure

Early nutrient restriction may permanently impair not only overall growth, but growth and development of specific organs that may contribute to long-term disease risk in later life. For example, there is a wide variation in the number of nephrons present in the kidneys at birth, and this number is then fixed for life. Reduced nephron reserve may increase the risk of later hypertension. Maternal nutrient restriction is known to impair fetal nephrogenesis in rats, mice, and sheep [12]. It also reduces angiogenesis and increases peripheral vascular resistance, all of which may contribute to offspring hypertension [30].

Similarly, cardiomyocyte proliferation is essentially confined to the prenatal period, with postnatal cardiac growth occurring by hypertrophy rather than hyperplasia. In rats, a maternal low-protein diet reduced cardiomyocyte numbers in the hearts of newborn offspring [31] and increased cardiomyocyte apoptosis in postnatal life associated with cardiac dysfunction [5]. Chronic protein-calorie undernutrition in rat dams also led to offspring with marked cardiac atrophy [32].

Likewise, most pancreatic β -cells are produced before birth or in the early neonatal period, and there is limited capacity for β -cell neogenesis after this time. Diabetes results when limited capacity to increase insulin production cannot meet the increased demand resulting for example, from insulin resistance, and is, therefore, more likely when pancreatic β -cell number is reduced. IUGR induced by maternal food restriction led to considerable reduction in β -cell mass in neonates and young rats [33]. Maternal food restriction in late gestation in mice also markedly reduced pancreatic β -cell mass at birth, and this relative reduction persisted into adult life [34]. When combined with insulin resistance, this would be expected to result in impaired glucose tolerance in later life.

Altered Placental Function

Maternal undernutrition may alter fetal growth and development by affecting the structure and function of the placenta. This may occur via changes in placental weight, histomorphology, vasculogenesis and angiogenesis, as well as placental nutrient transport capacity [35]. For example, in guinea pigs, maternal undernutrition led to increased placental barrier thickness and a considerable reduction in the surface area of syncytiotrophoblast for exchange, likely reducing the relative placental capacity to deliver substrates to the fetus [36]. In rats, maternal undernutrition led to enhanced apoptosis in the placental junction and labyrinth zones, the site for fetomaternal exchange [35].

Maternal undernutrition may not only affect placental structure, but also placental function. In rats, prolonged maternal malnutrition in late gestation

reduced circulating maternal glucose concentrations and the expression of GLUT3 in the placenta [37]. Protein restriction of rat dams downregulated placental amino acid transport, which appeared to be a possible cause of IUGR in these animals [38].

Altered Metabolic and Endocrine Environment in utero

The preimplantation embryo is particularly sensitive to epigenetic modifications that may have long-term consequences, and, as previously discussed, maternal protein undernutrition during the blastocyst stage of rat pregnancy led to hypertension in the offspring [19]. Kwong et al. [19] suggested that the transient mild hyperglycemia and amino acid deficiency in maternal serum due to dietary restriction may be a key underlying mechanism. However, subsequent embryo transfer experiments indicated that these effects were intrinsic to the blastocyst, rather than the environment in utero [39].

Nonetheless, there is evidence that undernutrition has a number of effects on the maternal physiological and endocrine milieu. In sheep, periconceptional undernutrition resulted in a delayed rise in early gestation progesterone concentrations in the ewe [22], and altered regulation of the maternal insulin/glucose axis that persisted beyond the period of undernutrition. Undernutrition before, but not after, conception also inhibited the normal development of the physiological insulin resistance of pregnancy in mid-gestation, which in turn was directly related to the growth of the fetus in late gestation (fig. 1) [40]. These and many other changes in the maternal physiological environment during pregnancy and lactation may be one mechanism by which relatively brief or specific changes in maternal nutrition can have long-term effects on the developing offspring.

Altered HPA Axis Function

Another mechanism by which early nutrition may have long-term effects on postnatal disease risk is by affecting the exposure of the developing offspring to glucocorticoids. Glucocorticoids affect growth and maturation of multiple tissues and, in particular, can lead to impaired growth, increased blood pressure and impaired glucose tolerance. These effects may occur in utero if maternal glucocorticoid concentrations are elevated, or if the placental barrier that protects the fetus from high concentrations of maternal glucocorticoids, mediated by activity of the 11β -hydroxysteroid dehydrogenase type 2 (11β HSD-2) isozyme, is impaired. Persistence of these changes after birth via permanent alterations in the regulation of the HPA axis in the offspring could explain many of the observed relationships between reduced size at birth and later disease risk.

In rats, intrauterine glucocorticoid exposure leads to reduced numbers of glucocorticoid receptors in the hypothalamus, resulting in impaired negative feedback and hence long-term upregulation of the HPA axis after birth [41].

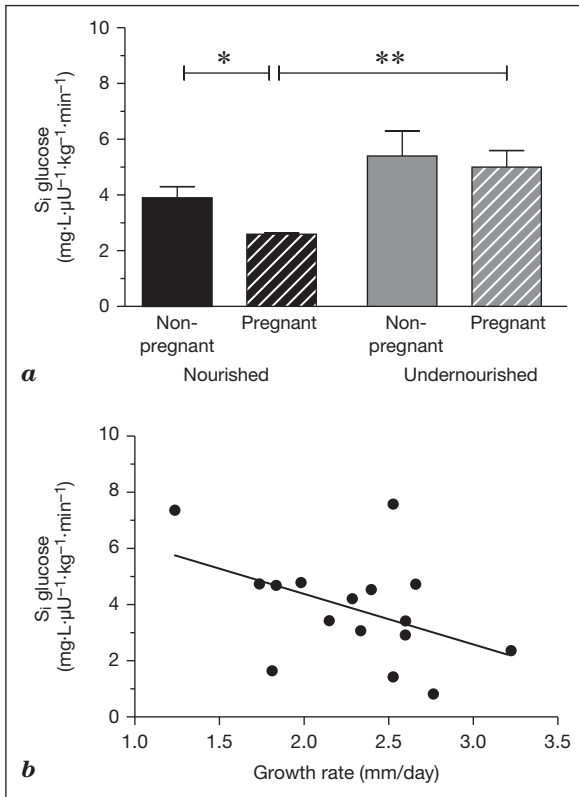


Fig. 1. a Insulin sensitivity (S_i glucose) in mid-pregnancy (65 days' gestation) in normally nourished and periconceptionally undernourished pregnant and nonpregnant ewes (* $p < 0.05$, ** $p < 0.01$). **b** Relationship between maternal insulin sensitivity in mid-gestation and fetal growth in late gestation.

This in turn could contribute to increased blood pressure and glucose intolerance in the offspring.

Maternal dietary restriction also increased maternal glucocorticoid secretion in rats, reduced placental 11 β HSD-2 activity [42], and altered neonatal HPA axis function. Prevention of the rise in maternal glucocorticoid concentrations by maternal adrenalectomy abolished the effect of a low-protein diet on the outcomes of interest in the offspring [43]. This provides convincing evidence that altering glucocorticoid exposure in early life may be one mechanism by which early nutrition can have long-term consequences for later health.

Maternal undernutrition in sheep also altered HPA axis function in the offspring before and after birth, even when the undernutrition was confined to

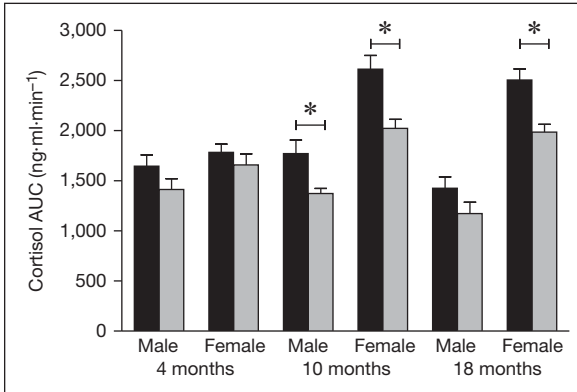


Fig. 2. Cortisol response to an AVP + CRH challenge in male and female sheep at 4, 10 and 18 months of age. Grey bars represent offspring of periconceptionally undernourished ewes, and black bars the offspring of normally nourished ewes. * $p < 0.05$ for nutrition effect. AUC = Area under the curve.

very early in gestation [22]. However, maternal nutrient restriction may not always result in a fetal environment of elevated glucocorticoid concentrations or in increased activity of the HPA axis after birth. Rather, sheep exposed to mild undernutrition for several weeks around the time of conception showed reduced basal and stimulated glucocorticoid concentrations, with altered regulation of the maternal HPA axis [21] and suppressed placental 11 β HSD-2 activity beyond the period of undernutrition [44]. Their offspring showed suppression of HPA axis activity that became more marked with age (fig. 2). Therefore, it is possible that the fetus of a chronically undernourished mother develops in a low, rather than high, glucocorticoid intrauterine environment, and that this can also result in long-term disease risk.

Altered glucocorticoid regulation of the anorexigenic hormone pro-opiomelanocortin (POMC) pathways in the ventral hypothalamus may be another mechanism by which fetal responses to maternal undernutrition result in an altered postnatal phenotype. In late gestation, fetuses of ewes undernourished from before conception until day 30 of a 148-day pregnancy displayed hypomethylation and increased histone acetylation of the promoter regions of POMC and the glucocorticoid receptor, 100 days after the undernutrition insult had ceased [45]. These epigenetic changes in the glucocorticoid receptor were reflected in increased gene expression [45]. In the ventral hypothalamus, the glucocorticoid receptor upregulates POMC. Thus, if persistent into postnatal life, these changes could result in disordered appetite regulation and contribute to the reported association between prenatal undernutrition and postnatal obesity.

Conclusions

Animal studies have provided an important contribution to our understanding of the mechanisms underpinning the long-term effects of reduced size at birth that have been observed in human epidemiological studies. Experiments in animals have shown that undernutrition in utero can result in growth impairment that is permanent, even if nutritional status subsequently improves. Observations in rats, mice, sheep, pigs and guinea pigs have also shown that the effects of undernutrition in utero may vary according to the nature, timing, severity and duration of the nutritional insult. Importantly, the long-term effects of early nutritional challenge can occur independently of size at birth, and even when the nutritional challenge is confined to the period before or around the time of conception. Some of the underlying mechanisms include impaired development of individual organs, altered placental function, altered metabolic and endocrine environment in utero, and altered function of the HPA axis and hypothalamic appetite regulatory centers. Clearly, nutritional quality and quantity in early life are critical in determining growth, development and disease risk for life.

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Discussion

Dr. Simmer: I would like to challenge you on one statement, and I think Peter Gluckman also has this theory that if you are well fed in utero you might be better off long-term to stay well fed. I don't think everyone agrees with that, I think there are other researchers in the literature that have challenged that assumption. Would you like to comment on that?

Dr. Harding: I think there is a good deal of confusion in the literature, and that understanding what is 'well fed in utero' is quite problematic. The limited animal data suggest that different outcome measures might give you different answers. In the example I showed you of maternal high-fat diet in rats, maintaining the offspring on a high-fat diet did not improve offspring blood pressure, but did improve endothelial function and oxidative status [1]. We also need to acknowledge that you might not be able to optimize all of the outcomes. It may be that if you are doing well for your brain, you are not doing so well for your heart or the other way round.

Dr. Agarwal: Do you know of any experiments with cereal proteins?

Dr. Harding: There are few data in animals, but I think it would be a very interesting area to investigate in more detail.

Dr. Pereira-da-Silva: I have a question and a comment. Regarding the maturation of the hypothalamic-pituitary-adrenal axis in the fetus, some animal studies suggest overexpression of neuropeptide Y in fetuses subjected to maternal undernutrition [2]. On the other hand, in fetuses growing under diabetic environment overexpression of the orexigenic neuropeptides Y and galanin and underexpression of the anorexigenic neuropeptide cocaine- and amphetamine-regulated transcript, persisting into adult

life, have been recorded [3, 4]. How important are these prenatal disturbances for hypothalamic appetite regulators in programming obesity? My comment is related to the long-term consequences of increasing nutrient intake in preterm infants, including by human milk fortification. The stimulating effect of milk protein on IGF-I secretion in early life, not seen with proteins of other origins [5], may be associated with adipogenic activity, increase in fat mass and programming obesity [6]. Now, you have presented a nice study showing that fortification of mother's milk (increasing the protein intake), increases the future lean mass in sheep. This may be good news for neonatologists who are concerned about providing better somatic and brain nutrition to preterm infants while avoiding programming obesity [7].

Dr. Harding: There has been a huge amount of work on how early nutrition changes appetite. There are major changes not only in neuropeptide Y, but in many of the hypothalamic appetite regulatory centers and signaling pathways [8, 9], and many of these changes have been shown to be epigenetically regulated [10]. How readily reversible those are is currently of great interest. In rats, for example, administering leptin in early postnatal life reverses most of the postnatal effects of maternal undernutrition [11]. Leptin is an important regulator of the neural connections in the hypothalamus in the early postnatal period in rats [12]. We don't know yet when that critical period is in humans, but this does point to the potential to reverse some of these effects.

Dr. Gottrand: I have a general question about the animal models of intrauterine growth retardation. Rats and mice have a very rapid growth during the first days after delivery, and some researchers consider that they could be a good model for studying late intrauterine growth retardation in humans for programming issues and so on. My question is, do you think that early undernutrition in the rat model or the mice model could be extrapolated to late intrauterine growth retardation in human and be used as a good model to test for programming or other issues about the impact of late nutritional defect?

Dr. Harding: One of the things that I learned as a PhD student from Geoffrey Dawes, who was the Doyen of Fetal Physiology, is that one should be careful in using the term 'model'. Animal studies tell you about animals, they don't model anything else. But, I do think you can learn what happens by looking for consistency across several species, and I have tried to show you that across a range of species similar things happen. So, having made that point, I am sure that early postnatal events in rodents are very similar developmentally and have similar long-term consequences to late gestational events in more mature species like sheep and humans, and I am sure that we can learn a great deal from influencing nutrition at that time. There is a large literature on the effects of changing nutrition during lactation in rodents, which is similar to changing nutrition in late gestation in more mature species. Similarly, many studies done in prenatal animals are very relevant to preterm infants because we are looking at similar developmental windows.

Dr. Lack: You showed on a number of graphs the separation between male and female animals, but didn't really comment on what the interactions might be between nutritional insults and sex.

Dr. Harding: Nearly all of the animal studies show sexually dimorphic effects, but they are not particularly consistent. The early and probably simplistic explanation was that the more rapidly growing animals, who are usually males, are more vulnerable to additional insults, be they pre- or postnatal, and therefore we saw greater effects in males than females [13]. I don't think that has held up with subsequent experiments, and we don't yet understand this inconsistency. However, this does suggest that in human studies we need to look carefully at different postnatal ages and in both sexes, when we are looking for long-term consequences.

Dr. van Goudoever: I was intrigued by the glycine aspect because I didn't hear anything about that before. What we did in studies in the preterm infants, especially in the growth-retarded preterm infants, we found that they were more likely to be glycine deficient than AGA infants. Can you relate that to any of your work which you have just been presenting? Is there a reason why especially SGA infants need more glycine than AGA infants?

Dr. Harding: Glycine is required for synthesis of things like heme and DNA and collagen, which are glycine rich and essentially end products in that the glycine cannot be recycled from these materials [14]. I could speculate that growth-restricted infants have a greater proportionate demand because they haven't made enough of these materials before birth. Whether this is causal or consequential, I cannot tell you.

Dr. van Goudoever: The other question I have is: how does high-protein diet affect blood pressure? How does it work?

Dr. Harding: There are numbers of possible mechanisms. Dietary protein can affect growth of nephrons and hence filtration load of individual nephrons [15]. It also can affect regulation of the renin-angiotensin system [16], impair endothelial function [17], and alter capillary density [18].

Dr. Rings: May I also add a question with respect to the blood pressure? I am so surprised that you see the effects of undernutrition so early in life, and my question is: would you advocate starting to lower blood pressure of these children as early as can be, like after birth? Would there be any reason to do that? And can you tell from your animal studies if interventions will be helpful to reduce the risk of cardiovascular diseases?

Dr. Harding: That is a really interesting question. The effects I showed you tend to be correlated with age, so the effects in childhood and early adulthood will tend to be small. They are, for example, much smaller than the effects of preterm birth. In our study, being born mildly preterm (median of 35 weeks) doubled the risk of hypertension by the age of 30 [19], whereas the effect of size at birth is in the order of 2 mm Hg/kg birthweight [20]. However, there is some evidence from the animal literature that early interventions may prevent the long-term changes. In rats, if the offspring of dams fed a low-protein diet are treated with an ACE inhibitor (for 3 weeks) in the neonatal period, they do not develop later hypertension [21]. But this is less a treatment effect than intervention during a critical window to reverse an earlier programming effect. It is like the effects of early leptin administration that we discussed earlier [11]. It suggests that if we understood the mechanisms and could identify the critical windows, we might be able to prevent some of the long-term effects.

Dr. Kleinman: That was an extraordinary review of a very complicated area, and I congratulate you on it. There are so many principles and mechanisms that this work reveals. At the same time, these findings suggest that pregnancy is an exceptionally sensitive and vulnerable period for the developing fetus. Can you comment on the threshold of dietary insufficiency here that leads to potentially harmful chronic health outcomes, because it doesn't seem like human biological systems would have evolved in such a vulnerable way to be so sensitive to the environment at this early stage of development.

Dr. Harding: I don't have an answer, but I have a couple of comments. One is the concept of the predictive adaptive response [22]. This proposes that the effects we are discussing are not evolutionary mechanisms, but rather adaptive mechanisms. During pregnancy, the fetus receives signals from the mother about the nutritional environment and makes the adaptations appropriate for that environment, so that the fetus is prepared as best it can be for the forthcoming extrauterine nutritional environment. Where the pre- and postnatal nutritional environments match, there is no particular health problem. It is only when you get a mismatch, for example when the offspring of

a small mother who is relatively undernourished grow up in a much better nutritional environment, that you start to see the kind of epidemics of diabetes and heart disease that we are seeing in some developing countries.

My second comment is that evolutionary explanations are unlikely to be helpful, because almost all of the relevant long-term diseases (hypertension, diabetes and so on) don't cause major morbidity until after childbearing, so that evolution does not act on those effects.

Dr. Giraldo: What is the impact of early nutrition on behavioral and neurological development?

Dr. Harding: Much of the animal work has been focused on changes in the HPA axis and related stress and anxiety responses. I don't think many of the animal studies are particularly helpful for understanding more subtle behavioral effects because of the relatively unique size, structure and timing of brain development in humans. We can show behavioral changes in sheep after periconceptual undernutrition [23], and others have undertaken behavioral studies in rats [24] and guinea pigs [25], but I don't know how relevant they are to human behavior.

Dr. Lack: The nutritional damage that is done to the offspring of the compromised mother, is that long-lasting into the next generation or is it done by the next generation?

Dr. Harding: There are many multigeneration effects. Altered maternal glucose tolerance in one generation affects pancreatic function in 2 or 3 generations, at least in rats [26]. Some of these effects can be passed through the paternal as well as the maternal line [27], suggesting that not all of the effect is via changes in the intrauterine environment. Rather, there are epigenetic changes in the early embryo.

Dr. Mohanty: I come from the part of India where infant mortality is the highest in the country, and 62% of that mortality is related to the neonatal period. People in India, especially the pregnant women, have a very low intake of food in terms of calorie, protein and fat. But we have a system that when there is a pregnant woman in the neighborhood, the relatives and friends invite her for dinner or lunch once or twice a month, and this goes on until delivery. What we find is that one third of deliveries belong to the low birthweight category, whereas two thirds are still appropriate-for-date babies. So the theory that the fetus is an obligatory parasite is true because the requirement of the fetus is quantitatively very small. So, I have two questions. Does the extra feeding that these ladies get during their pregnancy help them to recover and deliver appropriate-for-date babies, not very low-birthweight babies? Question two: is the theory of the fetus being a parasite irrespective of the protein and carbohydrate intake of the mother relevant today, since the food quantity required by the fetus is very small?

Dr. Harding: I don't know whether the extra feeding of the mother, even if it helps prevent growth restriction, is able to prevent the long-term consequences. The analogy in our sheep studies is of periconceptual undernutrition, followed by good nutrition for the remainder of pregnancy. We find that there is no effect on birthweight [28], but there are substantial changes in adult physiology [29]. Your second comment about the fetus being a wonderful parasite is an interesting one because you are right, the fetal nutrient demands, in absolute terms, are minute, particularly in early pregnancy. Nevertheless, those early pregnancy effects have long-term consequences. So, this story is not about deficiency of the nutrients required to build a fetus, and the fetus as an efficient parasite. Rather, it is about the way the developing embryo adapts to those nutritional signals.

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Dietary Lipid Quality and Long-Term Outcome

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Abstract

Understanding the importance of dietary fat has grown beyond energy metabolism to recognition of the complex roles of fatty acids, particularly the ω -6 and ω -3 fatty acids in membrane lipids, inter- and intracellular communication and in regulating gene expression. The ω -6 and ω -3 fatty acids accumulated in developing tissues depend on the fatty acids transported across the placenta and secreted in breast milk. These in turn are dependent on maternal fatty acid intakes, which have changed dramatically in the past century with current western diets high in ω -6 linoleic acid and low in ω -3 fatty acids. High intakes of ω -6 fatty acid and low intakes of ω -3 fatty acids compromise long-chain ω -3 fatty acid accumulation in tissues, and this is avoided by dietary docosahexaenoic acid. In addition to the well-known roles in neural development, newer studies are beginning to question the importance of ω -3 fatty acids as a contributor of metabolic development in other organs, with possible implications for the development of feeding behavior and integration of the nutrient energy supply.

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Introduction

Considerable evidence has accumulated to show that the nutrient supply in utero and during infancy has long-term implications for later development of cardiometabolic diseases, including obesity, type 2 diabetes and cardiovascular disease [1, 2]. Dietary lipids, specifically fatty acids have effects that extend beyond sources of metabolic and storage energy to central roles in the function of cell membranes, coordination of inter- and intracellular communication and as powerful modulators of gene expression. Central to concerns over the importance of dietary lipids in shaping early development, maternal diet is one of the most important variables contributing to the quality of fatty

acids transferred across the placenta and secreted in mother's milk [3, 4]. This review integrates knowledge of the essential and regulatory functions of fatty acids focusing on the ω -6 and ω -3 fatty acids to consider the importance of dietary fatty acid quality in early metabolic development and long-term outcome.

Dietary Fatty Acids and Their Sources

As introduced, maternal diet is one of the most important factors determining the types and amounts of ω -6 and ω -3 fatty acids transferred across the placenta and secreted in human milk. There is no doubt that the absence of Δ 12 and Δ 15 desaturase enzymes needed to insert a double bond at the ω -6 and ω -3 position, respectively, leads to essentiality of ω -6 and ω -3 fatty acids in humans and other animals. However, which and how much of the different carbon chain C18, C20 and C22 ω -6 and ω -3 fatty acids are needed in the diet remains a subject of uncertainty, further complicated by the impact of the dietary fatty acid composition itself on the metabolism of these fatty acids [5]. From a quantitative standpoint, the major dietary ω -6 and ω -3 fatty acids are the C18 linoleic acid (LA, 18:2 ω -6) and α -linolenic acid (ALA, 18:3 ω -3), respectively, with the richest source of these fatty acids being vegetable oils. Several of their C20 and C22 metabolites, including arachidonic acid (20:4 ω -6, ARA), adrenic acid (22:4 ω -6), eicosapentaenoic acid (20:5 ω -3, EPA) and docosahexaenoic acid (22:6 ω -3, DHA) are of particular interest with respect to neural and visual system development, regulation of gene expression, and inter- and intracellular communications. This includes the eicosanoids synthesized from C20 ω -6 and ω -3 fatty acids and the large family of acyl signal molecules, such as the acylglycines and ethanolamides [5–9]. In animals, including humans, LA and ALA provided in the diet can be converted to C20 and C22 metabolites in a complex pathway that requires Δ 6 and Δ 5 desaturases and several elongases (fig. 1). Conversion of LA to its metabolites ARA, 22:4 ω -6 and 22:5 ω -6, and conversion of ALA to EPA and DHA, however, is influenced by the amount and balance of LA and ALA in the diet. This is explained by the dependence of both LA and ALA on the same Δ 6 and Δ 5 desaturases for metabolism and the relatively low substrate needs of Δ 6 and Δ 5 desaturases for maximal activity [5]. Modern diets often provide high amounts of LA and are relatively low in ALA due to the types of fats and oils in the food supply [10]. The presence of preformed C20 and C22 ω -6 and ω -3 fatty acids in the diet is also important as the desaturation-elongation pathway is subject to feedback regulation. The C20 and 22 ω -6 and ω -3 fatty acids, mainly ARA, EPA and DHA are present in the diet in animal tissue fats, including human milk. While the amounts and types of ω -6 and ω -3 fatty acids in the diet and its impact on tissue lipid fatty acids is often considered with respect to human nutrition, it is important to

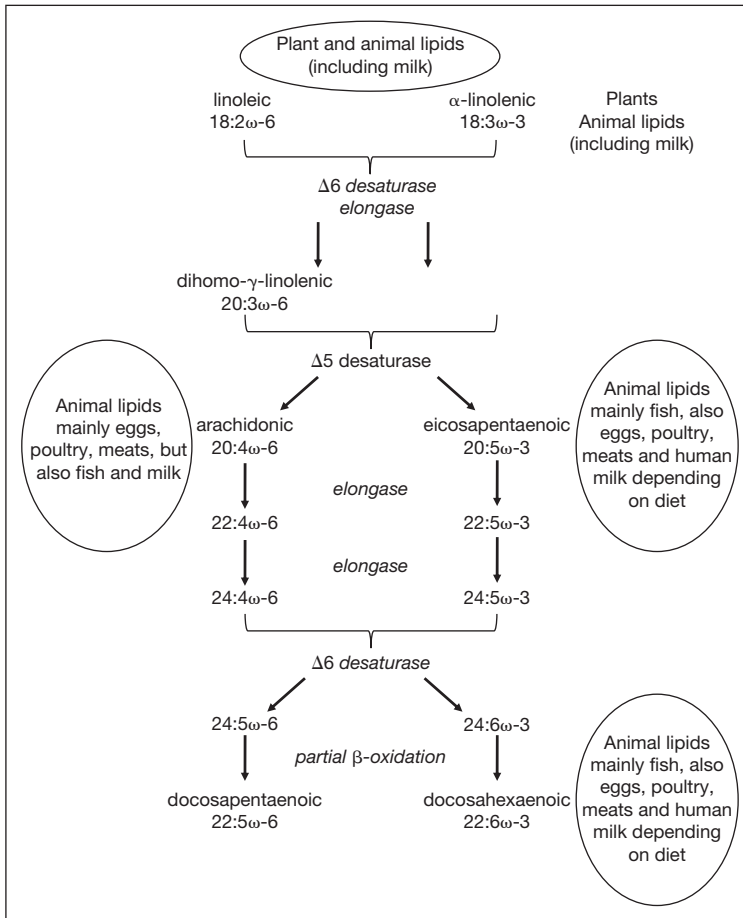


Fig. 1. Schematic of major steps of essential fatty acid desaturation and elongation with dietary sources of ω -6 and ω -3 fatty acids.

recognize that this also occurs in domesticated animals. Modern agriculture, including grain feeding, results in meats, poultry and eggs that are often high in LA and ARA, but low in ω -3 fatty acids, and this further contributes to higher intakes of ω -6 and loss ω -3 fatty acids [11]. Fish, in contrast to domesticated animals, are rich dietary sources of EPA and DHA, and this is explained by the synthesis of EPA and DHA in phytoplankton and their transfer up the aquatic food chain. Overall, from a practical perspective the types and amounts of vegetable oils and protein food choices (fish, meats, poultry and eggs) dictate the quantity and composition of ω -6 and ω -3 fatty acids in the diet.

Dramatic changes in dietary fat quality over the last century contribute to difficulties in understanding the dietary needs for ω -6 and ω -3 fatty acids, particularly of infants and the implication for long-term outcomes. Currently, LA provides 5% or more of dietary energy and 90% of polyunsaturated fatty acids in the average Western diet, while ALA provides about 0.5% of dietary energy [12]. Traditional diets consumed until the last 100–200 years, on the other hand, could have provided no more than 2% energy from LA, likely with equal amounts of ω -6 and ω -3 fatty acids and much higher proportions of total polyunsaturates from ARA, EPA and DHA [10, 11]. Two questions arise: the first is whether or not high intakes of LA flood the desaturation-elongation pathway making it difficult to synthesize long chain ω -3 fatty acids from the small amount of ALA in the modern diet; the second is whether humans have an inherently low capacity to desaturate and elongate ALA and require a dietary intake of preformed DHA, and perhaps EPA, for optimum health. Although it is well-known that vegetarians have lower blood lipid levels of DHA than nonvegetarians, explained by the differences in dietary DHA intakes, blood and human milk levels of ARA do not differ between vegetarians and nonvegetarians [13, 14]. While it is clear that large changes have occurred in the ω -6 and ω -3 fatty acids in human diets, much of the shift in dietary fat intake occurred with an emphasis in reducing elevated serum cholesterol [15] and without consideration of the potential impact of the ω -3 fatty acids or infant development.

Dietary Lipid Quality in Gestation, Breastfeeding and Early Infancy

A central question in understanding the physiological importance of maternal fatty acid nutrition for the fetus and breastfed infant is the extent to which selective placental fatty acid transfer or secretion in breast milk protects the infant from inadequate or inappropriate maternal fatty acid intakes. A large body of evidence shows that the quality of unsaturated fatty acids provided via placental transfer and secreted in breast milk is highly dependent on the maternal diet [3, 4], and this in turn impacts the fatty acid composition of fetal and infant tissue lipids [5]. Trans fatty acids are a clear example of the dynamic effect of maternal dietary fat quality on placental fatty acid transfer and secretion in breast milk, with levels as high as 12.8% trans fatty acids in plasma triglycerides of newborn infants and 18.7% trans fatty acids in breast milk of mothers consuming diets high in hydrogenated vegetable oils [16, 17]. Similarly, placental transfer and milk secretion of LA and DHA increase with increasing levels of LA or DHA, respectively, in the maternal diet [3, 4]. The mean levels of LA in breast milk fatty acids have doubled, from about 6–7 to 12–16% milk fatty acids, while DHA appears to have decreased by about 50% to a mean of 0.2–0.3% milk fatty acids in western

countries over the last 50–60 years [4]. However, the levels of DHA in human milk vary widely, both among and within populations, and the major reason for this is the dietary intake of preformed DHA. For example, breastfeeding women following vegetarian diets lacking DHA typically have 0.1% DHA in milk fatty acids, while women with high habitual intakes of fish often have 0.8% or higher DHA in their milk fat [4, 14]. The variability in DHA transfer across the placenta and in human milk has attracted considerable attention with respect to possible implications for infant development, particularly for the developing brain, retina and immune system [5]. However, another important question is whether concurrent exposure to high ω -6 fatty acid interferes with infant ω -3 fatty acid accretion. These two questions form the central dilemma in understanding fatty acid needs for optimal infant development and the implications of the fatty acid quality in current diets. It is well known that dietary ω -3 fatty acid deficiency leads to decreased DHA and a characteristic increase in 22:4 ω -6 and 22:5 ω -6 in the brain [5].

In this case, the 22:4 ω -6 and 22:5 ω -6 being formed from LA (fig. 1) [5]. In human infants, Farquharson et al. [18] reported levels of 17.7, 13.4 and 11.6% DHA and 3.2, 4.8, and 7.0% 22:5 ω -6 in cerebral cortex phosphatidylethanolamine of infants who had been breastfed or fed formula with 16.0% LA + 1.5% ALA or 14.5% LA + 0.4% ALA. The presence of high and increased 22:5 ω -6 in the brain of infants fed formula with very low ALA shows the desaturation-elongation pathway is active and raises the question of the importance of the dietary LA and ALA amounts. To address this question, we studied the effect of a formula diet with 1.2% energy from LA, which meets ω -6 fatty acid requirements for growth, or 10% energy from LA, with constant ALA, on ω -6 and ω -3 fatty acid levels in different organs of piglets [19]. The amount of 10% energy from LA was chosen because this is similar to the amounts of LA in some current infant formulae and also represents the upper end of the current recommended dietary intake range of LA in the US [12]. Comparison to a formula deficient in ALA enabled identification of the importance of ALA in restraining LA metabolism and preventing excess tissue accumulation of ARA. As shown in figure 2, after feeding from birth to 30 days of age, the ω -3 fatty acid (ALA)-deficient formula led to a marked decrease in DHA and increased 22:5 ω -6 and 22:4 ω -6 in cerebral cortex phosphatidylethanolamine, similar to autopsy data of Farquharson et al. [18], with a decrease in DHA and increase in 22:5 ω -6 in the liver and heart. The brain, in contrast to the liver and heart, is characterized by very low amounts of LA. Unlike the brain, ω -3 fatty acid deficiency is permissive for excess LA and ARA in both heart and liver. Importantly, DHA levels in the brain were reduced when the formula LA was increased to 10% energy, even though ALA was held constant at 1.1% energy (fig. 2). The capacity for accretion of very high amounts of EPA in the phospholipids of the heart and liver when the formula diet had a 1:1 balance of LA and ALA is remarkable, as is the loss of EPA when LA was increased to 10% of dietary energy. Overall, the data

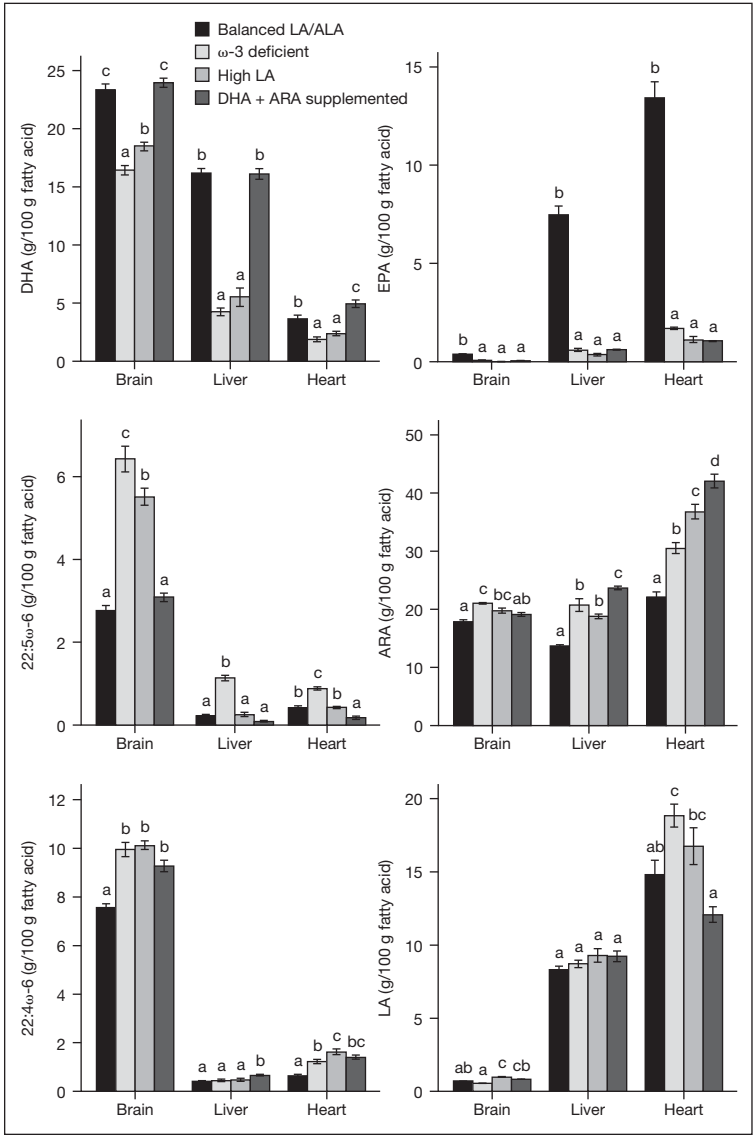


Fig. 2. Ethanolamine phosphoglyceride fatty acids in brain, liver and heart of piglets fed formula with, as percent dietary energy, 1.2% LA, 1.1% ALA (balanced LA/ALA); 1.2% LA, <0.1% ALA (ω -3 deficient); 10.7% LA, 1.1% ALA (high LA); 10.7% LA, 1.1% ALA, 0.3% DHA, 0.3% ARA (DHA + ARA supplemented). Values are means $\pm$ standard error of 5–7 piglets/diet. Bars with different superscripts are significantly different by ANOVA with Tukey's test for post-hoc analysis, $p < 0.05$.

indicate that the desaturase pathways are active and likely require very low amounts of LA and ALA substrate. Because human milk has LA of 6% fatty acids or higher, it seems likely that under practical circumstances; a dietary source of DHA is important for the developing brain. Indeed, as shown in figure 2, small amounts of DHA (0.3% energy) achieve high amounts of DHA in the brain. The importance of high amounts of EPA and the ARA/EPA balance in the liver, heart and potentially other organs still needs to be understood.

Early and Long-Term Effects of Dietary Lipid Quality

As discussed in the preceding section, developing infant tissues including those of the brain, liver and other organs are readily altered by the types and amounts of ω -6 and ω -3 fatty acids in the diet. A large number of studies in pregnant and lactating women, and infants fed formula have addressed the implications of the early DHA supply for visual and neurodevelopmental outcomes in infants. Recent reviews of these studies are available with the general conclusion that beneficial effects of DHA on visual and neurodevelopmental outcomes are more robust in preterm infants, with the findings in term infants inconsistent [20, 21]. A more recent area of interest is the possibility that early fatty acid nutrition, particularly the types and balance of ω -6 and ω -3 fatty acids may also impact development in other organs, such as the liver, and the potential for early programming of metabolic pathways, predisposing to characteristics of the metabolic syndrome [1, 2]. Two key areas of interest with respect to metabolic programming are the development of hypothalamic circuitry involved in the regulation of feeding behavior and metabolic programming involving altered expression of key genes and proteins regulating metabolic pathways in the liver [1, 2, 22, 23]. During short time windows in development, hormones and key metabolic cues are believed to play important roles in establishing the set point for receptor pathways and control of gene expression. Although it is known that the dietary ω -6 and ω -3 fatty acids impact ω -6 and ω -3 fatty acids in the developing brain and liver as shown in figure 2, little is as yet known with regard to the potential programming of neural feeding circuitry or metabolic development in the liver.

Mathai et al. [24] have provided evidence of long-term effects of ω -3 fatty acid deficiency in gestation and lactation on feeding behavior in rats. At 16 weeks of age, offspring of animals fed an ω -3 fatty acid-deficient diet showed increased food intake following appetite stimulation by food restriction or administration of the glucose antagonist 2-deoxyglucose, suggesting deficits in glucose regulatory appetite networks. Recently, we used 2-D gel proteomics to compare the entire protein complement in brain of embryonic and neonatal offspring of animals fed ω -3-deficient or adequate diets. Among several proteins responsive to ω -3 fatty acids, 14-3-3 protein zeta/delta was increased in the ω -3 fatty acid-deficient brain in both embryonic and 3-day-old neonates.

Notably, this protein is known to be increased in the brain in response to insulin [25], which in turn plays an important role as an early neurotrophic hormone controlling development of neurons in the hypothalamus [1, 22]. Further studies on the possible role of the early ω -6 and ω -3 fatty acid supply in the development of hypothalamic circuitry involved in feeding behavior are warranted.

It is known that ω -6 and ω -3 fatty acids have unique and important effects on energy substrate metabolism [6]. In the adult, ω -6 and ω -3 fatty acids are known to regulate several transcription factors including peroxisome proliferator-activated receptors, sterol regulatory element-binding protein, liver X receptors and hepatocyte nuclear factor 4, which control expression of genes for lipogenic, lipolytic and glycolytic enzymes [6]. Metabolism at birth is unique as the infant transitions from a low fatty acid supply in utero, representing about 11% total energy at term, to the milk diet which provides about 50% energy from fat, but is also relatively low in protein, representing about 8% of the energy in milk [4]. The transition from prenatal to postnatal life thus demands metabolic adaptation to maintain glucose and amino acids, while promoting fatty acid oxidation [26]. Studies have shown that maternal high-fat diets and protein deficiency alter key enzymes of hepatic glucose and fatty acid metabolism in the fetal and neonatal liver [23, 27], but relatively little is known about the importance of the ω -6 and ω -3 fatty acid supply in isoennergetic diets with constant protein, fat and carbohydrate. In recent studies, we used 2-D gel proteomics, together with targeted analysis of gene expression to find out if the maternal supply of ω -3 fatty acids impacts metabolic development in the offspring liver. Higher EPA and DHA in 3-day-old neonatal liver was associated with altered expression of proteins and genes not only for enzymes regulating fatty acid metabolism, but also glucose and amino acids; these included higher hepatic mRNA for carnitine palmitoyl transferase (*Cpt1a*) and acyl CoA oxidase (*Acox1*) and lower pyruvate kinase (*Pklr*), higher protein expression for glycerol-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase and serine hydroxymethyltransferase, lower argininosuccinate synthase and higher NADPH, as summarized in figure 3 [28]. The changes in gene and protein expression indicate that ω -3 fatty acids facilitate metabolic transition at birth conserving glucose for the pentose phosphate pathway leading to purine and pyrimidine synthesis and NADPH, sparing of protein from oxidation, and avoiding lipotoxicity by increasing fatty acid oxidation. While the long-term effects of the dietary ω -6 and ω -3 fatty acids on hepatic metabolism during development are not known, recent studies reported that a diet with 35% energy from fat with 18% energy from LA and 0.6% energy from ALA fed over 4 generations led to a gradual increase in fat mass due to combined adipose tissue hyperplasia and hypertrophy, with transgenerational alterations in adipokines, adipose tissue gene expression and hyperinsulinemia [29]. Given the role of ω -3 fatty acids in increasing fatty acid oxidation and of ARA-derived metabolites in adipocyte differentiation

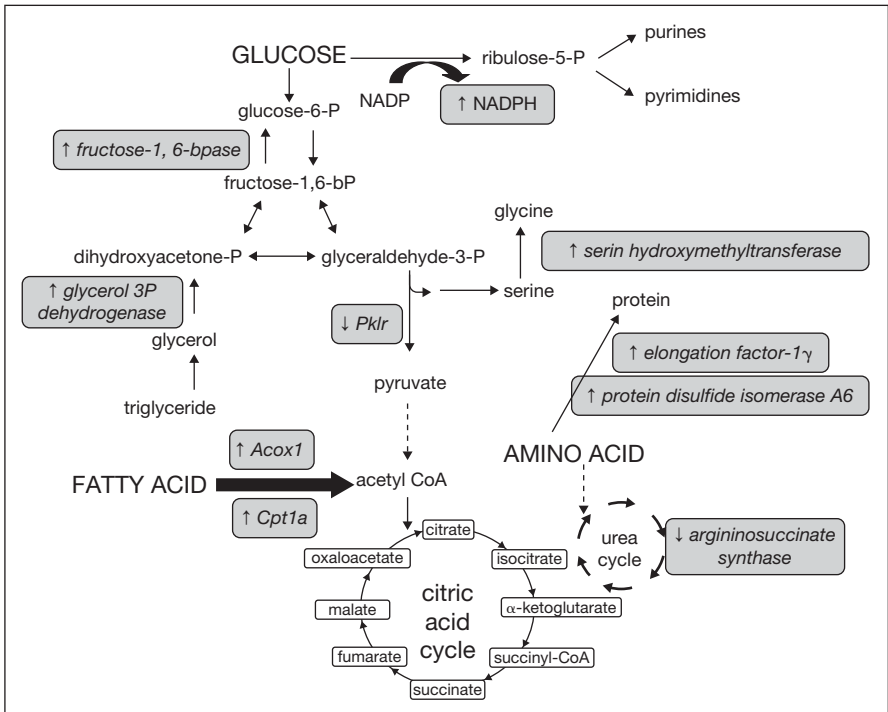


Fig. 3. Integrated schematic to show the results of proteomic studies, analysis of gene expression, and metabolites of livers from 3-day-old neonates born to rats fed an ω -3-deficient or adequate diet through gestation and lactation. Up and down arrows indicate higher or lower expression in the adequate compared to the deficient ω -3 fatty acid group. *Acox1* = Acyl CoA oxidase; *Cpt1a* = carnitine palmitoyl transferase; *Pkfr* = liver type pyruvate kinase.

via adipose-specific peroxisome proliferator-activated receptor- γ [30], future studies on the role of current westernized diets and the dietary fatty acid balance in early development of hepatic and adipose tissue development are worthwhile.

Conclusions

In summary, we have described the increase in ω -6 fatty acids in Western diets over the last century, and the potential loss of long-chain ω -3 fatty acids from the food supply. Changes in fatty acids in the food supply, including the increase in LA and decrease in ω -3 fatty acids are mirrored in the fatty acids transferred across the placenta and secreted in breast milk, thus impacting

not only the fatty acid nutrition of the mother but also that of the infant. The quality of the fatty acids provided to the fetus and infant impacts fatty acid accretion in multiple tissues, although in an organ-specific manner. While studies in this field are as yet limited, the role of ω -6 and ω -3 fatty acids as key regulators of numerous metabolic pathways, impacting gene expression and protein activities, suggests that further attention should be given to the possibility that the quality of dietary fatty acid in early life has both short- and long-term implications for human health.

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Discussion

Dr. Puri: I have two questions. The first one is: what should be the ideal ratio of omega-6 to omega-3 in the diet considering that vegetable oil consumption is more pronounced in countries like India? The second question is: is there any literature to support that omega-3 and omega-6 intakes in early infancy affect the manifestation of heart disease later on in life?

Dr. Innis: Following the argument related to the metabolic and biochemical physiology of humans, we know that dietary intakes of n-6 linoleic acid were low, and likely less than 3% of energy, until about 150 years ago. We also know that the amount of linoleic acid needed to saturate the $\Delta 6$ desaturase is very low, probably 1–2% of dietary energy. The pertinent question in our opinion is whether the amount of n-6 fatty acid or the n-6:n-3 fatty acid ratio is most important. Given the low needs of the $\Delta 6$ desaturase, our opinion is that the quantity of n-6 linoleic acid is more important than the n-6:n-3 fatty acid ratio.

There is no direct evidence in humans linking n-6 and n-3 fatty acids in early infancy to later heart disease. This is also difficult to assess because of the large changes in dietary fatty acids over the last half-century, with changes also including saturated and trans fatty acids. One of the major current concerns worldwide is the increase in metabolic syndrome. The n-3 fatty acids are associated with lower triglycerides, blood glucose, inflammatory mediators and possibly improved glucose tolerance. Whether this occurs in infants and can be programmed by the early n-6 and n-3 fatty acid supply is not known.

Dr. Gottrand: You nicely showed us that low omega-3 intake in the mother has an impact on the brain and on the liver. Are you aware of any work published on the gut in such models?

Dr. Innis: Yes. Studies from our laboratory have shown that the early fatty acid supply impacts intestinal development and susceptibility to later inflammatory responses [1, 2].

Dr. van Goudoever: Since DHA has such an effect on brain development and there is a wide variation in DHA in breast milk among different populations around the world, does that affect brain development around the world? Are there any data that the DHA content of breast milk impacts infant development?

Dr. Innis: Yes. Observational studies have linked higher DHA in milk or gestation to better child development [3–6]. However, there are no data to indicate that babies breastfed by vegetarian mothers have poorer outcome.

Dr. van Goudoever: The second question is related to my own field, neonatology, and it relates to your remark on DHA and the effect on neuronal migration. Preterm infants' neurons migrate from gestational age of about 24 to about 32 weeks, that's when all the neurons migrate. Should there be a role for DHA in that phase? What is your opinion?

Dr. Innis: Based on what we know about DHA and brain development, specifically the role in neurogenesis, it is reasonable to expect that the preterm infant will be much more susceptible to the effects of n-3 fatty acid deficiency than an infant born at term. Whether or not they need more DHA is a different question and we do not have that data.

Dr. Simmer: I wanted to make some comments about LA in humans. I think you suggested that in your piglets, reducing LA improved the DHA status. That's not what we find in preterm infants, and if we just talk about intravenously fed preterm infants where many people feed 100% soy oil, you can dilute that with olive oil or other oils, and you have absolutely no effect on DHA status of the preterm infant, so I am not sure the piglet is a great model. Then the other comment is about LA in the diets in general. I was at a fatty acid conference in Maastricht earlier this year, and there was a debate on the American Heart Association recommendation that you reduce LA in the diet, and it was the most aggressive unpleasant debate that I ever heard, and people are really strongly divided. I think it might be quite premature to on a population basis suggest that you reduce vegetable oils or LA in the diet. I think we need a lot more human randomized data before we can make recommendations about that.

Dr. Innis: In our piglet studies, we reduced LA to 1.2% of dietary energy, and with a similar amount of ALA, we showed that DHA was accumulated in the brain. Whether or not this would occur in humans consuming low amounts of LA, less than 3% dietary energy, is not known. Measurements of blood lipid fatty acids in premature infants supported by intravenous lipids are difficult to extrapolate to the brain; obviously, the fatty acids from the intravenous lipids exchange with the fatty acids in blood cells, and depending on when the blood sample is drawn, the plasma lipids may reflect the infusate if sufficient time for clearance is not allowed. You are correct, there is currently considerable debate regarding recommendations for linoleic acid. There is no doubt that it could be difficult, if not impossible, to consume 6–10% energy from polyunsaturated fatty acids without refined vegetable oils. We are of the opinion that if there is debate, there is no scientific consensus.

Dr. Klisch: You already addressed part of my question in terms of the ratio of omega-3 and omega-6 fatty acid. Because our population has become so dependent on plant oils, it's very hard to decrease the omega-6 component of that ratio. However, in infant formula, the solution has been to increase the DHA content rather than decrease the omega-6 or linoleic acid content. What would be more appropriate, knowing that

human milk is composed primarily of saturated fat and has only a small amount of unsaturated fat?

Dr. Innis: First, we do agree with the addition of DHA to formula. Infant formulas contain linoleic and α -linolenic acid, and now arachidonic and docosahexaenoic acid. Human milk, however, has more than two n-6 fatty acids and more than two n-3 fatty acids. Understanding of the importance of fatty acids such as 20:3n-6, 22:4n-6 and 20:5n-3 in organs other than the brain is limited. We would not recommend reducing linoleic acid in current formulas until more is known.

Dr. Fasano: I would like to pick your brain about a strong debate far to be settled concerning maternal nutrition and, therefore, fetal development and mental performance. I was very intrigued by the data you mentioned about the maternal high-fat diet and metagenomics and methylation of genes. You state that dopamine definitely affects behavior; share with us your thoughts about that, and what you really think this would imply in terms of performance in young kids based on fetal nutrition.

Dr. Innis: In our experience of working with pregnant women, poor dietary n-3 fatty acids, specifically DHA intakes, are often associated with different dietary patterns and lower intakes of several nutrients than in women with high intakes of DHA. In addition, DHA is associated with protein in foods, since it is found only in animal tissue lipids. It seems likely that the association between low DHA or fish intake in pregnancy and child outcome is complex, and may well involve several nutrients. Animal studies have shown maternal nutrition impairs gene methylation in the offspring; these studies, however, are primarily high fat/low fat (low carbohydrate/high carbohydrate) comparisons.

Dr. Fasano: Actually, what I was getting at was, do you think that poor nutrition, either malnourishment, undernourishment or unbalanced nutrition, puts the kids in disadvantage in terms of social intellectual performance.

Dr. Innis: Yes, but this will depend on the severity, duration, the nutrient in question and timing in pregnancy when the nutrient deficiency or excess occurs.

Dr. Kleinman: What is the maximum ratio of linoleic to linolenic acid, before you inhibit the desaturases?

Dr. Innis: Based on what we know about the desaturases, these enzymes appear to be fully saturated with substrate. As I mentioned earlier, it seems more likely that the quantity of fatty acid is important; changing a ratio from say 10:1 to 4:1 would not make any difference if the enzyme is saturated.

Dr. Kleinman: Infant formula appears to meet that optimal ratio. Thus, if desaturases are active even in fetal life, it's a little hard to imagine why there would be a need to supplement that system with polyunsaturated long-chain fatty acids. I also have a comment about these functional cognitive and behavioral outcomes. As I interpret the published data, differences in outcomes converge over time, so that by the age of 8 or 10 years you can't separate the groups by type of early infant diet. Even during the periods of time when there are outcome differences, as you pointed out, these are inconsistent and are often present at different time points, in different studies. Thus, there doesn't seem to be a lasting effect or potentially even a short-term benefit that leads to a lasting effect from early fatty acid supplementation. This conclusion seems also to be supported by the wide variation in the concentrations of long-chain polyunsaturates in breast milk. There is as much as a 20- to 40-fold difference in concentration of omega-3 long-chain fatty acids from one country or region to another. Thus, it doesn't seem to follow, from an evolutionary perspective, that it would be necessary to supplement either the pregnant mother or the young full-term infant with LC-PUFAs.

Dr. Innis: It is correct that current studies relating fatty acids to infant outcome are inconsistent, and that long-term outcome data are lacking. A main point of our piglet study was the demonstration that taking a diet with high linoleic acid and adding

arachidonic acid and docosahexaenoic acid does not give the same tissue lipids, for example in the heart, as a diet with low linoleic acid. We agree that there is still much to be learned about the desaturases and pathways of phospholipid fatty acid acylation.

Dr. Simmer: Having written the Cochrane reviews on this, I think you are absolutely right for the term infant, but for the preterm infant a big Australian trial which was published in *JAMA* last year showed that high-dose DHA did reduce disability, but it was looking at the question of in utero supply compared with the breast milk supply for babies <30 weeks gestation, which is a different scenario.

Dr. Mace: When we talk about the benefit of omega-3 fatty acid, we always talk about DHA. What about α -linolenic per se? What are the known biological effects of α -linolenic acid?

Dr. Innis: This is a very important point. It is simplistic to think about n-3 fatty acid requirements simply from the perspective of DHA. For example, α -linolenic acid with a low linoleic acid diet clearly functions to restrain linoleic acid metabolism. Whether or not α -linolenic acid has unique functions is unclear; however, given the very different metabolism for α -linolenic acid, this is a reasonable hypothesis.

Dr. Shreffler: I was intrigued by the systems biology/proteomics approach you took, and would like you to defend that a little bit. How do you go about validating those targets and choose which outcomes to correlate targets to? Do you see consistent changes, for example with omega-3 deficiency that you do with maybe omega-6 excess, etc? And do changes on the transcriptional level corroborate your protein changes?

Dr. Innis: We chose the systems biology approach because little is known about the developing liver, and the infant milk diet with 50% energy from fat is clearly very different from the adult diet. For the proteins that showed a change in abundance, we mapped these onto metabolic pathways and then looked for corroborating evidence to indicate that pathways were altered through gene expression using real-time PCR. For example, we have looked at carnitine palmitoyl transferase and acyl-CoA oxidase (fatty acid oxidation) as well as pyruvate kinase (glycolysis).

Dr. Shreffler: And if I could add, how well do changes correlate for example with varying those ratios, you know where you see other similar outcomes?

Dr. Innis: We have not yet looked at hepatic protein or gene expression with adequate n-3 but varying n-6 fatty acids.

Dr. Guandalini: I was intrigued by the data you presented on maternal high-fat diet influencing the choices of food in the offspring via programming of opioid receptors. Now, if that is true in the humans as well, that would really be important in terms of health policy. It seems to me that we are creating a vicious cycle by having a high-fat diet during pregnancy thus predisposing these children to our choices which are unhealthy in terms of excess fat. What is your comment on this?

Dr. Innis: This is a fascinating hypothesis. Studies are currently underway in our lab to look at this.

Dr. Zlotkin: This question is more for Dr. Kleinman and Dr. Simmer. In South Asia, a third of the infants born are low birthweight, some of those are preterm and some of them are low birthweight, non-preterm. Most of the data in the Cochrane reviews are from developed countries on both preterm and full-term infants. I would agree with your conclusion in terms of the functional impact. For the infants born for example in south Asia who are low birthweight, do you think that the conclusions that have been drawn from the meta-analysis of studies in the developing world would also fit?

Dr. Innis: I would agree, it is not correct to extrapolate from current meta-analysis to low-birthweight infants in South Asia.

Dr. Kleinman: I think that when you are dealing with a population that begins with a diet that is severely restricted in calories, protein, fat and quality of fat and very

far from what we know to be optimal and you are also dealing with an infant who is born very prematurely, it makes biological sense that you need to support that in different ways than what we have been talking about. There aren't a lot of data though to help us answer this question.

Dr. Stathatos: You mentioned that high fat diet decreases the methylation process. I would like to know whether the high fat in your studies was unsaturated fat or saturated fat. And the second question is: what is the effect of saturated fat on long-term health processes?

Dr. Innis: The studies showing altered methylation in animals fed different diets are important because they show the biological plausibility and importance of methylation in developmental programming. However, studies thus far linking diet in animals to methylation are difficult to extrapolate to humans. For example, 40% energy from fat and high saturated fat is unphysiological for rodents. Very little has been done on saturated fat and early human development. As we know, human milk is 20–25% palmitate, representing about 10–12% of the infant's energy intake. We have no indication that the saturated fat in human milk has any untoward effect.

Dr. Kleinman: Could I ask a follow-up question on the opioid receptors and the increased perception of sweet taste? We know that we are born with an innate preference for sweet and salt. Can diet enhance those preferences or influence the way they change over time?

Dr. Mennella: You don't have to teach children to like sweet or salt; that, as you said, is innate; they are born preferring a much more intense sweetness, and this doesn't decline until around mid- to late adolescence. There is some work to suggest that when the sweet preference starts decreasing, it is like the closing of the epiphysis. So, there may be some metabolic factors signaling back to the taste receptors. There are individual differences, and one of the things that we have been looking at is the brain pathways related to the actions of drugs and abuse which basically just co-act with the pathways that were designed for sweet, so the opioids and the dopamine. We find that children who have a family history of alcoholism or addiction prefer a much higher level intensity of sweetness as young as 5 years of age. So, one way of looking at it is, the mouth is really an indicator of how the brain is processing these hedonic signals. Some of Liem's and Gerry Beauchamp's work are showing that what children learn is the context of what food should be sweet. That's the cultural learning, how sweet should something be. There has been no work for both salt and sweet to show how one can shift them downward. Whether lowering the sodium content of the diet will lead to a global shift downward in preference is a good question. No one knows. When we think about sweet for children, it's not just the tasting, sweets make children feel good. It's a very powerful stimulus that we have now in our environment, where we have refined sugars, we have intensities of sweetness that we probably have never encountered before. These are all important questions that really haven't been looked at.

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How Proteins Improve the Development of Preterm Infants

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Abstract

Amino acids and proteins play a pivotal role during growth and development. Besides acting as building blocks during tissue synthesis, amino acids or proteins act specifically by upregulating defense systems or by stimulating key sites in metabolic pathways. Following premature birth, the neonatologist is responsible for delivering the right amount and quality of nutrients to the neonate, while exact requirements are largely unknown. However, nutrition matters, both in quantity as well in quality, especially during the first few weeks and months of life. It is increasingly recognized that proteins and amino acids in the immediate postnatal phase have both short- and long-term influence on later life.

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Introduction

Major innovations in perinatal care, like antenatal steroids and improved respiratory support, have improved the survival rate of preterm infants remarkably. This increase in survival has, however, not been accompanied by a similar decrease in morbidity, although for instance cystic periventricular leukomalacia has almost disappeared [1, 2]. Adverse neurodevelopmental outcome is still substantial [3, 4] and improvements in the field of nutrition do not seem to have resulted in a lower incidence of postnatal growth failure [5–7] or substantial improved outcome [8–9]. As stated recently by the ESPGHAN Committee on Nutrition, the goal of caring for premature infants is obtaining a functional outcome comparable to infants born at term [10]. Thus, it is mandatory to optimize neonatal nutrition in such a way that unhampered

brain growth and development is stimulated. In the field of nutrition, amino acids or proteins have, apart from specific bioactive functions, a central role as they form the matrix of all new tissue and are thus responsible for growth. Therefore, an overview of several aspects of amino acids and proteins in neonatal research will be presented.

Nutrition for Premature Infants – A Brief History

Apart from its relevance in pediatrics, long ago the importance of the newly recognized substance protein was immediately acknowledged. The word protein was coined by the Swedish chemist Jöns Jakob Berzelius in a letter sent in 1838 to his Dutch research associate Gerhardus Johannes Mulder who first documented its chemical structure. Berzelius wrote: 'The name protein that I propose for the organic oxide of fibrin and albumin, I would like to derive from *πρωτεϊος* [proteios] (meaning of the first rank or position), because it appears to be the primitive or principal substance of animal nutrition' [11].

Later, in the early 1900s, Pierre Budin, a French obstetrician, together with his mentor Stéphane Tarnier, the founders of modern perinatal care, provided a clear statement on neonatal nutrition. Budin wrote: 'The path of pleasure, for adults, is drinking. May it not be the same for weaklings? I increased their absorption of milk with, as you have seen, the happiest of results' [12]. Thus, in those days premature infants, called weaklings, received high volumes of human milk by tube feeding (up to 200 ml/kg per day) to stimulate rapid growth [13]. However, from the 1940s onwards, concerns about aspiration pneumonias and kidney failure resulted in withholding all fluids for up to 72 h after birth. Up until approximately 1965, very little attention was paid to nutrition. This changed once it was recognized that adverse neurodevelopmental outcome could be attributed to low initial fluid and nutrient intake and early provision of fluids/feedings was again advocated [13]. Since then, several small adaptations to formulae or breast milk fortifiers have resulted in the current methods used to feed babies enterally [14].

Intravenous nutrition also has a long history. The first report on intravenous amino acid administration to young infants in 1939 described many complications [15]. More triumphant was the report that appeared in 1944 where a marasmic suckling received solely total parenteral nutrition for 5 consecutive days [16]. Almost 25 years later, a low birthweight neonate with near-total small bowel atresia received total parenteral nutrition for 44 consecutive days without any enteral feedings; her weight increased by 80% during the study period [17]. Besides stimulating growth, these first solutions containing hydrolyzed amino acid residues also caused significant problems such as hyperammonemia [18]. After the introduction of synthetic crystalline solutions, other undesirable effects such as acidosis became apparent [19].

These findings, together with a report that very high enteral protein intake (6.0–7.2 g/kg per day) in infants born below 1,300 g resulted in lower IQ scores at age 5 [20], still have a profound effect on current nutritional policies. Although it was recognized that withholding amino acids resulted in a catabolic state, they were withheld during early life under the assumption that the preterm infant was ‘intolerant’ to amino acid solutions. We have come to realize that both the method of manufacture and the composition of the amino acid solutions were likely to have caused complications such as hyperammonemia and metabolic acidosis, rather than the amino acids solutions *per se*. Nevertheless, fear of metabolic derangements is still firmly rooted in clinical practice.

Fetal Nutrition

Whether fetal nutrition can serve as a model for neonatal nutrition for premature infants is a difficult question. Certainly, postnatally there is a different physical environment often complicated by disease and medical interventions. Besides, waste products such as ammonia cannot be excreted anymore through placental removal. Yet, for example, metabolite concentrations in fetal plasma provide a safe threshold as to which postnatal values can be referenced. Additionally, fetal enzymatic activity and metabolic rates may indicate metabolic capacities at a certain gestational age which should also pertain to the newborn of similar age. Third, the fetal nutrient deposition during normal growth, provide the minimum amount of nutrients that is also necessary after birth to support a similar growth rate. Studying fetal metabolism can also give good insight into the differences between intrauterine growth restricted infants and those normally grown. Unfortunately, knowledge on fetal nutrition, metabolism, and growth remains scarce, especially in humans [21–23].

From fetal studies, largely performed in sheep, we have come to learn since long that, for example, large amounts of amino acids are actively transported across the placenta towards the growing fetus. These rates well exceed those necessary for tissue deposition or growth [24, 25], and are used as additional fuel source as also demonstrated by large urea formation [26]. These observations, for example, have now contributed to a higher targeted protein intake in premature infants [10].

A different area in fetal research pertains more to developing an effective antenatal therapy for fetuses with intrauterine growth failure, also in humans. Enteral and parenteral nutrient supplementations to the mother have mostly been unsuccessful or even contraproductive [27, 28]. Imbalanced diets together with reciprocal placental transporter inhibition are at least partially causative. More invasive and direct attempts have also been made by intra-amniotic infusion of nutrients [29]. Infection risk and potential induction of

preterm labor make repetitive clinical implementation improbable. Recently, however, a case report was published where a port system was implanted subcutaneously to gain permanent access to the umbilical vein [30]. This enabled chronic fetal parenteral nutrient supplementation after which successful fetal growth acceleration was claimed. Apart from being uncontrolled experiments in which fetal growth is hard to follow longitudinally, many lessons from animal research have also been learned. Not only is nutrient status usually compromised during fetal growth failure, optimal transplacental oxygen delivery is for example also crucial for ongoing metabolism. Direct umbilical nutrient infusion can thus result in worsening acidosis with adverse outcome.

Neonatal Nutrition

After birth, the nutrient supply through the umbilical cord stops abruptly. Very low birthweight (VLBW) infants are then dependent on externally administered nutrition while their endogenous nutrient stores are very limited. Without adequate nutrient supply, protein breakdown will increase, resulting in a catabolic state. Although it was already stated in 1977 by the American Academy of Pediatrics Committee of Nutrition that a premature's postnatal growth rate should duplicate fetal growth rate [31], this goal is still not achieved most of the time [5, 6, 32]. Note, however, that initial postnatal weight loss is also due to excreting excess extracellular water and not solely catabolism. Energy and protein deficits develop mainly during the first 2 postnatal weeks when parenteral nutrition is not initiated at target intakes and tolerance of enteral substrates is low [33]. These deficits prove hard to recoup.

Causes of inadequate nutrient intake include fear for intolerance of parenteral and enteral nutrition. Also, fluid intake is restricted to minimize complications such as patent ductus arteriosus and chronic lung disease. Third, acute neonatal illness such as ongoing sepsis or necrotizing enterocolitis (NEC) as well as metabolic derangements such as hypertriglycerolemia or severe uremia all result in a reduced nutrient administration. Aside from stagnation of somatic growth, other short- and long-term adverse effects from undernutrition are not encountered instantly. However, growth failure also reflects overall underdevelopment of many organs which has life-long consequences for the functioning of these organs [34], thereby making the individual more prone to diseases such as diabetes and cardiovascular diseases. Short-term consequences of under- or malnutrition can result in increased vulnerability to infectious diseases [35], higher susceptibility to lung injury caused by impaired tissue repair and muscle weakness [36], and decreased maturation of intestine [37, 38] or brain [39].

Parenteral Nutrition

During the last decade, several studies have shown the beneficial effects of early parenteral amino acid administration; it reverses a negative nitrogen balance towards anabolism, and it increases plasma amino acid concentrations towards reference ranges found in fetuses or healthy neonates [40–42]. Possible involved pathways, other than providing anabolism, include reduction in oxidative stress by upregulating glutathione synthesis rates [43]. In addition, the increased synthesis rate of e.g. albumin following amino acid administration, may be responsible for an increased transport and binding capacity, with a reduction of potentially toxic levels of free bilirubin or medicines [44]. However, the exact amount and composition of nutrients, required by premature neonates for optimal growth and development, remains unknown [45]. In some neonatal intensive care units (NICUs), amino acids are infused to premature infants from birth onwards, but elsewhere more than 36 h are awaited before commencement of parenteral amino acid administration. Also starting doses vary widely between different NICUs; 0.5 g/kg per day followed by a stepwise increase to 2.5 g/kg per day, but also 3.5 g/kg per day at once. An awaiting attitude results from fluid limitations, risk of hyperglycemia in the case of mixed glucose/amino acid solutions and fear for intolerance, although no firm ground exists for this approach.

Over the years, the quality of intravenous amino acid solutions has improved. Nevertheless, the fear for intolerance in premature infants is still deeply rooted. Safety of amino acid administration is clinically mainly based on biochemical parameters, such as acidosis and plasma concentrations of urea, ammonia, or individual amino acids. However, none of these parameters are specific for amino acid intolerance, and all are influenced by the clinical status of the neonate as well.

Te Braake et al. [40] for example showed that VLBW infants fed glucose and amino acids (2.4 g/kg per day) from birth onwards did not have clinically relevant aberrations in acid-base status. Urea concentrations were, however, significantly raised in the intervention group (9.6 ± 2.8 mM), but no potential side effects of increased urea concentrations at these ranges have been reported [46]. Others did not observe a correlation between amino acid intake and acid-base status [42, 47, 48] or uremia [42, 49] at all. However, Blanco et al. [50] infused extremely premature infants (25.7 ± 2.0 weeks' gestation) with high-dose amino acids soon after birth (up to 4 g/kg per day on day 3 of life). Whereas the mean peak urea concentration was already very high (19.6 ± 6.8 mM), it even ranged up to 36 mM in some of the most immature infants (≤ 24 weeks). Ammonia concentrations were also elevated in these infants (~ 100 mM), where normal values during early life in fasting premature infants are 70 ± 25 mM [51] and a very wide range can be measured in cord blood [52].

Effects on anthropometric measurements are not consistent among studies on early amino acid administration. Valentine et al. [53] observed a greater

weight gain till discharge in premature infants where amino acids were started within 24 h after birth. Poindexter et al. [8] also found in a large observational study a greater weight, length and head circumference at 36 weeks postmenstrual age in those neonates who had achieved an amino acid intake higher than 3 g/kg per day before day 5 of life. At 18 months corrected age, no differences could be detected anymore apart from a larger occipitofrontal circumference in boys only. Also no differences in the mental and psychomotor indexes, as well as the occurrence of handicaps between both groups were observed. Stephens et al. [54] on the other hand, found that in extremely low birthweight infants, after adjusting for confounding variables related to disease, an increase of 1 g/kg per day of protein intake during the first week of life was associated with an 8.2-point increase in mental developmental index. Higher protein intake was also associated with a lower likelihood of length <10th percentile, but not weight or head circumference, at 18 months corrected age. Ehrenkranz et al. [9] showed that a higher growth velocity during NICU admission has a significantly, and possibly independent, effect on growth and neurodevelopment at 18 and 22 months corrected age. The rate of weight gain was significantly and inversely related to the likelihood of bodyweight and length below the 10th percentile around 20 months corrected age, but not related to the likelihood of head circumference below the 10th percentile. Nevertheless, it was shown that with increased weight gain and increased growth of head circumference, the incidence of cerebral palsy, mental and psychomotor developmental index scores <70, and abnormal neurologic examinations fell significantly.

In conclusion, most data provide some evidence for the beneficial effect of rapid initiation of relatively high-dose amino acid administration to the average premature infant.

Enteral Nutrition

Nowadays in most NICUs, trophic or minimal enteral feeding (MEF) is initiated directly after birth (e.g. 1.5–3 ml, six times daily). These small volumes of milk are nutritionally insignificant from a nutritional view, but are thought to stimulate maturation of the developing gut. Infants given MEF show enhanced activity of digestive enzymes increased digestive hormone levels and improved gut motility when compared to infants who do not receive MEF. Infants given MEF tolerate full enteral feeding earlier, without increased incidence of NEC [55]. Enteral nutrition can be given in the form of human milk (mother's own milk or pasteurized human donor milk) or as (preterm) infant formula. Since the first commercially available infant formula in 1915, much research has been devoted towards the development of a formula that resembles the composition of human milk as much as possible and evokes similar physiologic responses in infants. The proteins in formula are most often derived from cow's milk. An important difference between human milk and cow's milk protein is the whey-to-casein protein ratio. Bovine milk has a whey

content of approximately 20%, whereas human milk has a whey content of 80% (early lactation) to 50% (late lactation). This has important implications for the amino acid profiles that become available after degradation of milk proteins. For protein synthesis to proceed at optimal rates all essential amino acids must be present in the diet in appropriate amounts. Also, as the brain relies on one single amino acid transporter for all large neutral amino acids to be transported across the blood-brain barrier, imbalances in blood amino acid profiles may also lead to differences in brain amino acid concentrations which could result in altered neurotransmitter concentrations. If and how this would affect the formation of synapses and differentiation of brain cells in the developing brain is not yet elucidated.

Human milk is the optimal nutrition for healthy term born infants, and it is assumed that fortified human milk is also the optimal nutrition for preterm neonates. An additional important difference between human milk and cow's milk is the lower availability of cysteine and tryptophan in cow's milk, and therefore these amino acids may become limiting in a diet based on whole cow's milk. This difference in availability is partly explained by differences in whey-to-casein ratios between human milk and cow's milk. Specific formulae, such as whey-enriched and α -lactalbumin-enriched formula, have been developed to more closely resemble protein and amino acid profiles of human milk.

Many human milk proteins not only supply the amino acids for protein synthesis but also serve as biologically active components. Human milk proteins can for example stimulate intestinal maturation (growth factors), aid in nutrient absorption (e.g. bile salt-stimulated lipase) and provide protection against pathogens (e.g. immunoglobulins). Some proteins might exert their effect as signaling molecules or might affect through modulating the intestinal flora or immune system. A part of the biologically active components of human milk can be synthesized by e.g. recombinant techniques or gained from cow's milk and added to formula. Some of them retain their activity and have shown to be of benefit for the preterm infant when added to artificial formula (e.g. lactoferrin, which might reduce the incidence of late-onset sepsis [56]) whereas other constituents (e.g. IGF-I [57]) do not seem to be of any advantage to preterm infants if administered outside the matrix of human milk.

There is debate on the usability of some of the biologically active proteins, as they have been found intact in the stools of breastfed infants, and consequently on the true protein intake of breastfed (preterm) neonates. Lactoferrin and sIgA are quantitatively the most significant (together 0.2 g/100 ml in mature milk) of the relatively indigestible proteins. It has been estimated that 6–10% of lactoferrin escapes digestion by breastfed infants [58]. This would be a potential loss of 0.012–0.02 g of protein per 100 ml, and therefore the effect of the loss of these amino acids on the protein intake of infants is likely to be insignificant. These amounts might be higher in the

premature neonate. Another important difference is the high non-protein nitrogen (NPN) content of human milk (20–25% of total nitrogen) when compared to the NPN content of milk from other species (usually <5%) [58]. The NPN fraction consist of entities like free amino acids, peptides and about 50% is urea [59]. The urea in human milk might be used by colonic bacteria for the synthesis of amino acids that become available for the host. To what extent this contributes to bioavailability of amino acids most likely depends on the colonization pattern of the host, which is severely disrupted in the premature, partly formula-fed, antibiotic-treated infant admitted to the NICU. From adult studies, we know that a significant part of the essential amino acid requirements are provided by intestinal bacteria [60]. Therefore, when thinking about the nutritionally available protein or amino acid content of human milk, the less digestible proteins and the high NPN content should be taken into account.

Human milk itself generally does not contain enough protein (and energy) to meet the high demands of the VLBW infant. Multinutrient supplements are composed of extra protein and carbohydrate, but also vitamins and minerals are added. Recent studies show that infants fed fortified human milk still often receive less protein than they actually need and less than is assumed by their physicians. The reason for this discrepancy is that manufacturers of breast milk fortifiers designed their products to fortify preterm milk with an average protein content of 1.5 g/100 ml. Assumptions that all preterm milk has an average protein content this high is unjustified. Although preterm milk might have a higher protein content during the first weeks of lactation, this declines within a few weeks to the level of term milk, amounting on average to 1.2 g/100 ml [61]. Fortifiers, when prepared according to the manufacturer's instructions, add on average 0.8 g of protein per 100 ml of milk. To reach the same protein levels as in preterm formula (2.5 g/100 ml), the unfortified milk must thus contain 1.7 g/100 ml, a level that will not be met in most cases. A possible solution to this problem could be titration of extra protein to human milk on the basis of regularly determined biochemical parameters such as serum urea [62]. However, it is debatable what would an appropriate parameter be and what reference values should be used. Another solution, but labor intense, is bedside measurement of protein content by the use of easy to operate human milk analyzers and to adjust the addition of fortifier to the found values. However, weight gain rates using such a personalized approach do not show a great improvement over standard fortification [62].

By adding a fortifier to human milk, however, one must note that cow's milk protein is introduced into the infant's diet. Based on the observation that there is a higher incidence of NEC in formula-fed infants, it can be hypothesized that this is not due to protective effects of human milk but rather to a sensitizing or disrupting effect of milk (protein) derived from cows [63]. With relatively simple techniques like ultrafiltration and freeze

drying, it is possible to gain human milk proteins from donor milk [64]. This, although requiring financial investments and additional training of staff, makes production of a human milk-based fortifier possible for human milk banks. It can be hypothesized that this could have beneficial effects such as lower incidence of sepsis and NEC. But also improved neurodevelopment in the long-term can be expected as a consequence of amino acid profiles more suited for the human neonate. However, this needs to be confirmed in large randomized trials. Several studies so far have examined the use of an exclusive human diet. It was shown that addition of a human milk-based fortifier did not result in altered growth or blood parameters of protein status when compared to a bovine fortifier [64]. A recent study suggested a reduction in NEC incidence when the infants were put on an exclusive human diet [63].

Nutrition and Epigenetics

Premature infants are born at a time which, in utero, is characterized by rapid brain and body growth. As discussed above, often nutritional and therefore growth targets are not met. Fetal and early postnatal life is characterized by a high plasticity. During this phase, 'signals' from the environment may induce changes in the expression of the genome and thereby permanently changing the phenotype of the organism. This capability of adjusting to the environment is probably limited to a critical period in early life and is followed by loss of plasticity and fixed functional capacity. If the resulting phenotype is well adapted to the future environment, this may confer a fitness advantage. However, 'erroneous' signals or assaults in early life may give rise to a phenotype that is more prone to disease [65]. Nutrition is an important link between the environment and the (developing) organism, and evidence is accumulating that early nutrition is strongly influencing the risk of adult-onset diseases, such as diabetes and obesity. The hypothesis is that when an organism is malnourished during fetal or early postnatal life, it anticipates to receiving a low nutrient supply in later life by adjusting the setting of hormones and metabolism. The molecular basis for this kind of adaptations to the environment is only partially understood. It is thought to be the result of an altered *expression* of the genome caused by changes in DNA methylation and covalent modification of histones ('epigenotype'). Alternations in epigenotype are therefore seen as the molecular basis for the long-term effects of early nutrition. Additionally, as nutrition is a key factor for normal cell growth, poor growth in early life may also directly result in a fewer number of cells in key organs, thereby compromising organ function. Early disrupted growth and formation of pancreatic islets of Langerhans are, for example, an easily conceivable concept to eventually result in diabetic mellitus.

Conclusions

We here presented a broad overview of some aspects of the role of amino acids and proteins in human neonatal nutrition and how these might affect outcome. Although it is well known that proteins and amino acids have a central role in growth and development, large randomized controlled trials remain sparse in order to tailor nutritional intake optimally towards unhampered growth and development. Nevertheless, a lot of progress has been made over the years. More evidence is accumulating that rapid provision of parenteral nutrients in the immediate postnatal phase benefits the prematurely born neonate. However, the long-term outcome of early nutritional interventions with substantial power mainly comes from observational studies. Concerning enteral nutrition, the beneficial role of breast milk is increasingly recognized over that of formula feeding. Its specific protein content with several bioactive proteins is not fully present in formula. An important disadvantage of feeding premature neonates with human milk is the low nutrient content, which is at present only partly solved by the addition of a milk fortifier. However, current available methods for fortification leave room for improvement.

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Discussion

Dr. Stettler: Can you speculate on the mechanism that underlies the difference between boys and girls in response to protein intake in the first 2 days?

Dr. van Goudoever: It's a very good question. What we see in all kinds of nutritional trials in preterm infants, and this also goes for the 6 or 7 trials on post-discharge formula that are out there, that there is a gender effect. There is a gender effect that boys are sensitive to nutritional change, whereas girls are much less sensitive to changes in nutritional regimen. Also with regard to example given glutathione synthesis rates, we find these differences. So on all different kinds of levels (body composition, weight gain rates or metabolic pathways) we see differences in gender, and they are always directed towards an effect, an improvement in effect in boys, whereas hardly any effect in girls. But again, all the major negative outcomes, such as necrotizing enterocolitis or BPD – they are more frequently observed in boys than in girls.

Dr. Stettler: I am wondering whether understanding better what the mechanism is could lead to therapeutic implications.

Dr. van Goudoever: We may end with different kinds of nutritional interventions for boys and girls because sometimes we have a hint, also in our new study on parenteral intakes, that actually girls are worse off with higher protein intakes than boys. So, if you perform an intervention for the whole group, you might see no effect, whereas on average boys are doing better and girls are doing worse. I think that at present the results our trials should always make a distinction between boys and girls.

Dr. Haschke: A practical question related to amino acid composition of breast milk versus cow's milk or cow's milk-based formula. If premature infants receive milk from their own mother, how will the next generation of human milk fortifier look like? Should it be an amino acid supplement or a supplement based on cow's milk protein fortified with the necessary amino acids?

Dr. van Goudoever: There are two explanations why formula-fed infants have more necrotizing enterocolitis than infants fed mother's milk. One is that there is a protective effect of human milk, the other one is that there is a detrimental effect of intact cow's milk proteins. In this context, does the quality of human milk versus formula milk depend on whether you fortify it with a bovine milk protein, a hydrolyzed bovine milk protein, completely extensively hydrolyzed amino acids, or should you supplement mother's milk with a human milk-based fortifier? The result from the Sullivan study actually suggests that cow's milk protein might be detrimental. Coming back to your original question (regarding the different requirement of the amino acids), we are still far away from knowing each individual amino acid requirement to know exactly what preterm infant needs. The majority of studies performed so far have just been looking at total protein and maybe protein energy to quality ratio, and not so much at the individual quality of proteins.

Dr. Chittal: The data are convincing that only protein intake is good for growth and also the neurodevelopmental outcome, but these early protein intakes are also

causing increased blood pressure even at 7–8 years. So where is the exact balance of protein intake?

Dr. van Goudoever: Very good question, and I don't know. The only argument I have is that we have to make a distinction between preterm and term infants. Preterm infants are at risk for having severe neurocognitive impairment, and that should be addressed first before I would bother too much about higher blood pressure or outcomes like that. What I think we are doing in our wards and in our step-down units is actually underfeeding these infants, we are not giving too much. By increasing their protein intake you might be right that this would lead to higher blood pressure rates. However, and this relates back to Dr. Haschke's question, we have to consider the quality of the proteins as well. You can give lots of protein, but growth rate is limited by the first limiting essential amino acid in your diet. So, if you are low on one specific essential amino acid, you can put in as much other amino acids as you want, the one that is the limiting one is limiting your growth and also your organ growth and also your nephron growth and brain growth, etc.

Dr. Saluja: In the NICU, most of the preterm neonates get about 2.5–3 g of proteins as a fortification of their formula. What should be the policy for those preterm neonates who move on to direct human milk or direct breastfeeding in the post-discharge phase? We know that they may not be able to consume enough proteins. If we want to follow their growth velocity in terms of weight, length and head size, what should our targets be? And how should we customize their intakes to match their growth references?

Dr. van Goudoever: Again an excellent question, and we are focusing all the time on weight gain rates because that's easy to do. But Prof. Harding already showed that not only weight gain or rather anything else than weight gain might be important if you are interested in long-term consequences. Still, there is not that much we can measure in daily care, so then probably weight gain measurement is the easiest thing to do. The infants who are on human milk should receive fortifiers. A Danish study has been published where infants fed fortified breast milk only once a day with additional proteins displayed an increase in weight gain rates in the post-discharge phase or in the late hospital phase. I don't know whether fortifiers are available in India, but supplementing human milk with fortifiers once or twice a day would be an option.

Dr. Saluja: We do have a fortifier, but unfortunately the quality and amount of proteins it provides are inappropriate. Is it fair to say that when we follow the infants' growth, we should continue to follow the same centiles as in the prenatal period, and that if they are growth restricted we don't allow them to catch up and cross the centiles?

Dr. van Goudoever: The catch up is again an important question. In my view, what is happening in the first couple of weeks to months is that we underfeed our infants and they become growth retarded. In my unit in Rotterdam, the average birth z score was –0.8, and at discharge they reached –1.6. The majority of that loss is in the first few weeks. In my view, if we are able to feed them appropriately in those first few weeks, which is really much more than we do now, then the whole question about detrimental catch-up growth is futile. So, I think the key is within the first few weeks of life.

Dr. Pereira-da-Silva: I have a practical query. You suggested that a daily intake of 4–5 g per kg of bodyweight of amino acid might be appropriate for the very premature babies. This exceeds the current recommendations, at least for parenterally fed premature infants [1]. Which short-term risks should we expect when administering excessive doses of amino acids to these infants?

Dr. van Goudoever: Basically, the newest recommendations are 3.5 g of parenteral amino acid intake and up to 4.5 g of enteral protein intake [2], and the difference

is of course caused by the utilization of amino acids by the intestine. What are the effects of a high amino acid/protein intake: (1) hyper ammonia, (2) high amino acid levels with detrimental effects, and (3) high urea levels which might cause kidney problems later on. Of course, there have been studies in the 1970s with high, very high (up to 5 or 6 g) levels of amino acids. There have also been studies on parenterals with high amino acid levels, but in that case there were no crystalline solutions, for instance, so they had all kinds of detrimental effects. I think quality is the subject that we should pay a lot of attention to in the next 5 years, and maybe we can even lower total protein intake again by improving the quality, by improving let's say the first 3 or 4 limiting amino acids in the diet. If we can increase those intakes, we might even decrease the total protein intake.

Dr. Pereira-da-Silva: And guided by serum ammonia and urea, is it possible to push the intake over the currently recommended dose?

Dr. van Goudoever: Yes that's actually what we do nowadays, we measure urea levels. Based on urea levels, we have set the maximum cord blood level at 10 mM, and we basically titrate amino acid intake based on that level.

Dr. van Elburg: You were talking about amino acid quantity and that you might differentiate between the different amino acids that are added to the infant's diet. Based on your stable isotope studies, which amino acids would you increase and which ones would you decrease?

Dr. van Goudoever: It's interesting, across all mammals lysine and threonine are the first two limiting amino acids in the diet, so those are the two that are limiting for enteral nutrition. Actually, the pig industry has known that for a long time, they add lysine into pig food and the piglets grow faster. For parenterals, I don't know exactly. I know that Paul Pencharz in Canada is doing lots of studies on parenteral amino acid intake requirements, but mostly in older children, and those studies need to be done as well.

Dr. Iramain: We know that in preterm newborn a daily intake of 4 g protein/kg can cause renal failure. How can we approach this issue?

Dr. van Goudoever: I agree; that's why I say let's monitor some short-term metabolic outcomes like urea levels. But then again, what would be the most important improvement you want to see in preterm infants? I think that the most important improvement we would like to see concerns neurocognitive development, and I think that is something that we should strive for. The amino acid intake through the umbilical cord is about 5 g/kg per day, but maternal metabolism will take away all metabolites.

Dr. Mace: What about the body composition of these infants? Don't you think that fat mass is also something important to consider?

Dr. van Goudoever: I completely agree. Again, it's hard to measure fat mass in the NICU. In our new studies, we give doubly labeled water to first measure energy expenditure, but we also get fat mass. So, it needs to be done; it's exactly what you say, it's not only weight gain rates but it's also body composition.

Dr. Fasano: I was intrigued by your concept of the challenge of neonatologists to recreate the in utero nutritional environment for the very low-birthweight infants and the debate cow's milk versus breast milk versus placental nutrition and so on and so forth; but I would like to know what your thought is about the fact that now you have a different situation because the intestine is very different in utero (sterile) than in an NICU where you have colonization. But I am assuming all these kids are on antibiotic treatment. Does this change the dynamic of the game that you are going to play in terms of nutrient utilization?

Dr. van Goudoever: It certainly did. I was very reluctant to make big steps in trying to improve the nutritional management until I had the data about what the infants

were receiving in utero. Recently, we designed a trial which is currently running with larger steps, looking at long-term outcomes with 240 kids, for instance.

Dr. Were: I was intrigued by your studies telling us that if you delay introducing proteins as early as the first or second day you lose time, and the dose that you build up slowly from 1.1 up to 4.5 has worse outcomes. It would require having in-house facilities for parenteral nutrition to start feeding infants early. What advice would you give to the centers like the one I come from where we don't have those facilities? How can someone in a little corner of Malawi be able to attempt to get close to giving 2.4 g on day 1 to a 1.2-kg baby?

Dr. van Goudoever: It's a good question, a question which I have been asked frequently in Southeast Asia as well. The question is where do you put your money? Do you put your money in a new ventilator or do you put your money in getting parenteral nutrition in your unit? It's all about costs, how much resources you have, to what extent you can convince the director of your hospital that nutrition, although it seems so simple, has such a major effect on long-term outcomes. Of course, it's not easy to accomplish parenteral nutrition in Malawi for premature infants. But if we can convince people that this is a very important issue, that although nutrition is not very fancy, it really can change late outcome in many lives, we are on the right track. We need to convince key opinion leaders in the different hospitals that early nutrition has a major impact, and I think that the time now is right to do it.

Dr. Agarwal: Why don't we give pregnant women amino acids in the later part of pregnancy? You give them infusions at the recommended doses before the cesarean section. Maybe this way the preterm delivery would also be prevented.

Dr. van Goudoever: That's a good question as well. There have been studies in the 1970s of actually providing pregnant women in New York with high levels of protein, but the outcome was worse: higher infant mortality, higher number of premature births, and so basically nobody dares to do those kinds of studies again. Infusion of amino acids in growth-retarded infants didn't work. So, there are a lot of questions there. It seems simple, and I completely agree, but it doesn't seem to work.

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The Knowns and Unknowns of Human Milk Banking

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Abstract

The provision of donor human milk instead of formula is an important contribution to the nutrition and protection from infections for preterm infants. Systematic reviews suggest a lower risk of necrotizing enterocolitis with pasteurized donor human milk (PDHM) as opposed to artificial formula, although evidence supporting PDHM use from randomized control trials is limited. Human milk banks (HMBs) must have a risk management system to maintain a safe product especially as many operate in an unregulated environment. To ensure safety, the HMB in Australia has committed to meet the appropriate standards recommended in the Code of Good Manufacturing Practices (Blood and Tissues) and models risk management during processing on Codex HACCP (Hazard Analysis Critical Control Point) requirements. There is scope to continually reevaluate the screening of donors and quality standards recommended during HMB. This will be most effective if strong networks of HMBs are developed with regional reference laboratories to encourage compliance with safety guidelines. Further research and development is needed to refine technology for treating donor milk such as thermal ultrasound and ultraviolet light, aimed at the retention of full bioactivity. HMB networks will facilitate collection of evidence for refining HMB practice which should translate to improved outcomes for preterm and sick infants. Cost effectiveness is most likely when HMBs are associated with large neonatal intensive care units.

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Introduction

Human milk (HM) performs the dual function in all mammals, providing protection and nutrition for the young. The primary advantage of mammals is their ability to reproduce and nourish their young in any environment that supports the adult. Evidence suggests that the nutritional components

evolved from the innate immune system [1]. The innate immune system is an evolutionarily non-antigen-specific, host defense system that provides immediate protection against invasive microorganisms. HM has many important immune properties, for example TLR (Toll-like receptor)-mediated innate immune responses are specifically and differentially modulated by HM [2]. TLRs are ancient innate immune receptors that are expressed by most human tissues, and are crucial for the recognition of pathogen-associated molecular patterns [3].

Provision of donor HM for preterm infants when mothers' own milk (MOM) is not available is preferable to formula but pasteurization reduces the immune and nutritional benefits to variable degrees. The only human milk bank (HMB) in Australia was established in July 2006 with the opening of the Perron Rotary Express Milk (PREM) Bank at King Edward Memorial Hospital in Perth, Western Australia (WA). Previous efforts at HM banking ceased in the mid-1980s with the identification of transmission human immunodeficiency virus (HIV) via HM. After 4 years experience at the PREM Bank, we have had the opportunity to review our own data and re-assess what is known and remains unknown with regard to HM banking.

Somewhat to our surprise, we have quickly discovered that the community fully support HMBs. The PREM Bank was initially established with all equipment purchased from community donations. Media interest is limitless and almost exclusively positive. We have also found that mothers experiencing an oversupply actively seek us out and, as such, donations have always exceeded our processing capacity. We now also know that neonatal staff prefer to prescribe pasteurized donor HM (PDHM) rather than formula, as shown by steady increase in use since the milk bank opened in WA. The number of babies receiving donor milk has doubled over the past 3 years, but the duration of feeding and the average volume dispensed to each infant has remained reasonably constant. In 2009, the PREM Bank dispensed PDHM to 211 patients for an average of 15 days each. In 2009, 92 mothers donated 1,482 liters of milk. We now know that parents of preterm infants welcome PDHM when MOM supply is inadequate with only a few mothers in WA declining the options of donor milk instead of preterm formula (PTF).

We also know that our experience is not unique and that the World Health Organization and UNICEF have jointly supported the establishment of HMB as part of international efforts to promote and support breastfeeding: 'Only under exceptional circumstances can a mother's milk be considered unsuitable for her infant. For those few health situations where infants cannot, or should not, be breastfed, the choice of the best alternative – expressed breast milk from an infant's own mother, breast milk from a healthy wet nurse or an HMB, or a breast milk substitute fed with a cup, which is a safer method than a feeding bottle and teat – depends on individual circumstances' [4]. HMB is also referred to by the American Academy of Pediatrics [5] as a suitable alternative when an MOM is insufficient or unavailable. Despite the long history of

HM banking, evidence from randomized clinical trials (RCTs) is limited and includes trials in India by Narayanan et al. [6], in England by Lucas et al. [7], and in USA by Schanler et al. [8].

Narayanan et al. [6] randomized preterm infants to raw or pasteurized HM $\pm$ formula and demonstrated pasteurization reduced the protective effects of HM (14.3 infection vs. 10.5%), but infants fed PDHM had lower infection rates than those fed formula (33.3%). Lucas et al. [7] randomized 502 infants weighting <1,850 g PDHM or PTF and demonstrated similar developmental scores (Bayleys) at 18 months which they interpreted as PDHM conferring advantage that was offset by the relatively deleterious effects of low nutrient content. The authors concluded that it is logical to combine the benefits of HM with that of extra nutrition provided in fortifiers. Schanler et al. [8] randomized infants 23–29 weeks' gestation being fed MOM, to PDHM or PTF once tolerating >50 ml/kg per day if MOM supply was inadequate. Intention-to-treat analysis with 21% PDHM being switched to PTF for poor growth and infants still receiving 50% MOM, showed no benefit of PDHM in primary endpoint of late-onset sepsis and/or necrotizing enterocolitis (NEC). The study design and sample size have been criticized, and the authors agree further studies are required. Meta-analysis of randomized trials of PDHM vs. PTF found a lower risk of confirmed NEC with PDHM (as entire feed RR 0.25, 95% CI 0.06–0.98, as supplemental feed RR 0.3, 95% CI 0.11–0.87) [9].

It is unlikely that future evidence for HMB will come exclusively from RCTs of PDHM. Clinicians working in neonatal intensive care units (NICUs) with access to donor milk may have ethical difficulty randomizing high-risk patients to artificial formulae where the risks are known, and where their own clinical experience suggests fewer complications when donor milk is used. Because these PTF are constantly changing, it could always be argued that to ensure scientific rigor, RCTs would need to be repeated regularly to evaluate potential improvements. We suggest that it is time to accept the evidence of potential and reasonable clinical benefit of donor HM for preterm and ill hospitalized infants. The evidence to date carries enough weight to encourage the establishment HMBs where they are managed to an appropriate standard. We also propose that it is the responsibility of these donor HMBs and the NICUs to which they provide product, to engage in research to better assess potential benefits of donor HM and improve the products provided by HMBs.

What We Know about the Benefits of Pasteurized Donor Human Milk

For preterm infants [9, 10], PDHM reduces the incidence of NEC 4-fold and improves feed tolerance. This may be associated with reduced days of parenteral nutrition and earlier discharge from hospital. Pasteurization reduces the protective effects of HM, but feeding PDHM is associated with a

lower incidence of infections than feeding formula [6]. It is also known that preterm infants fed PDHM grow less well than those fed MOM [8, 10], but the significance of this is unclear. It is unknown whether the high IQ scores associated with feeding MOM to preterm infants [11, 12] relate equally to PDHM. For term infants, there is very limited evidence for benefit. HIV-negative infants, of HIV-positive mothers, fed PDHM had, in general, a larger thymus than infants of healthy mothers fed formula interpreted by the investigators as indicating a benefit due to immunomodulatory factors in breast milk [13]. PDHM has been used successfully to treat short gut syndrome [14]. There is no evidence to support the higher IQ with breastfeeding of term infants [15, 16] relate also to PDHM. There is no evidence that breastfeeding or feeding PDHM is useful in the prevention or treatment of CMP allergy [17, 18]. Breastfeeding is one of the few preventative measures for reducing childhood obesity rates [19], and this may be due to the lower protein content of HM [20]. There is little data on the body composition of infants fed PDHM, although there are theoretical reasons for a potential benefit of feeding PDHM instead of formula in reducing childhood obesity. It has been suggested that feeding PDHM improves quality of life for pediatric and adult patients with cancer [21], short gut syndrome, postsurgical feeding problems and numerous other conditions based largely on anecdotal evidence [22]. We conclude that there is some evidence of benefit for preterm infants (reduced NEC risk, decreased sepsis and neurodevelopment), and therefore these patients should remain the focus of donor HM banking. We are concerned that making donor milk available to outpatients or otherwise healthy term infants may result in donor milk becoming another alternative to mothers feeding their own infants. Conclusive RCT evidence in support of PDHM use in preterm infants seems unlikely, and focusing on collecting other physiological measures, for example ultrasound to assess gastric physiology, may be a better use of resources.

What Do We Know about Human Milk Fortifiers Produced from Human Milk

Sullivan et al. [23] randomized 207 infants with birthweight <1,250 g in the USA to three groups. Two groups received only HM (MOM $\pm$ PDHM + HM-based fortifier starting at 40 or 100 ml/kg per day milk feeds), and one group received HM + bovine products (MOM $\pm$ PTF + bovine milk-based fortifier). Infants fed HM + bovine products had a higher incidence of NEC than those fed exclusively HM products. There were no differences in feed tolerance or growth between the groups. The rate of NEC in the HM + bovine products was relatively high compared to contemporary outcomes. The bovine products included formula and fortifier. The HM-based fortifier is commercially available from a private company in the US.

The Safety of Human Milk Banks – Management and Regulation

HMBs should be managed and regulated in such a way as to ensure that appropriate measures are undertaken to allow response to unforeseen risks. In the mid-1980s when HIV was identified in HM, most HMBs closed. In Australia, HMB management at that time did not allow a rapid response to this unforeseen risk. The informality of the screening process and the lack of complete record keeping, donation traceability and document and process control were insufficient to respond to this new threat. Since the emergence of these new diseases, similar industries have developed management strategies to allow public confidence in the safety of these valuable services. Blood/tissue banking and the food industry have risk management tools (e.g. Australian Code of Good Manufacturing Practices – Blood and Tissues and Codex HACCP, Hazard Analysis Critical Control Points, requirements) that can be adapted for use during HMB management.

Many countries continue to struggle to maintain the credibility and confidence of clinicians in milk banking safety and efficacy due to the lack of a regulatory body governing the operation of HMBs [24]. In countries such as Brazil, where governments have specific legislation regulating milk banking, these difficulties appear to be significantly reduced [25]. During the establishment of the first HMB in Australia, we encountered barriers as existing legislation did not recognize HM as a Therapeutic Good or Food [26]. Thus, the two bodies regulating the production of these products, the TGA (Therapeutic Goods Administration) and FSANZ (Food Standards Australia New Zealand) did not have a legal framework to regulate HMB. Each State has control over food manufacturing. In WA, the PREM HMB was established with a rigorous quality and safety system based on HACCP. Governments in the eastern states of Australia have not yet endorsed HMB. As part of the current Australian government's response to the 'Best Start Report' [27], the Australian National Breastfeeding Strategy (2010) was developed which recommends national regulations for HMBs be developed based on the WA guidelines. These new national regulations will also be consistent with the Biological Tissue Framework [28]. It is highly unlikely that it will be an option in Australia to feed raw donated milk. Regulation will mandate that the milk be treated to manage the risk of viral transmission and to reduce bacterial contamination.

The Safety of Human Milk Banks – Processing of Donors and Donations

In general, HMB in most countries commit to screening milk donors for the same blood-borne viruses as required by blood banks. Rationalization in some centers has led to dropping of screening for HTLV and restriction

of hepatitis B screening to surface antigen. HTLV is destroyed by pasteurization and freezing, and false positives are associated with influenza vaccination. In Australia, women who have lived for 6 months or more in the UK between 1980 and 1996 are excluded as breast milk or blood donors because of the risk of transmission of variant Creutzfeld-Jakob disease, while countries immediately affected by the bovine spongiform encephalopathy epidemic continue to operate HMBs without evidence of harm. Other reasons for exclusion of donors include an assessment of any medications or pharmacologically active herbal products a donor mother may be taking that may be transferred to breast milk. Much is known about the transfer of common medications into breast milk, and exclusion of donors based on maternal drugs is rarely necessary but proceeds on a case by case basis [29–31].

Bacterial cultures of milk are not consistently performed in all HMBs, and the bacterial count limits for rejecting milk vary between HMBs and from that recommended [32]. In Australia, and most countries, neonatal units do not routinely culture or pasteurize MOM. The risks of feeding heavily contaminated MOM to very preterm infants are unknown. Law et al. [33] cultured 10,128 samples of MOM from 96 mothers fed to infants with birthweights <2 kg in the first 2 weeks of life. They detected no growth in 19% of samples, only Gram-positive bacteria in 74%, only Gram-negative bacteria in 1% and mixed Gram-positive and -negative bacteria in 6% of samples. Gram-negative bacteria were present in at least one sample of milk from 51 of 96 mothers' milk (53%). There were few adverse events that could be related to ingestion of bacteria in raw milk, but colonization of the gastrointestinal tract by Gram-negative species may have occurred after ingestion of the same species in 48% of 62 babies exposed [33]. Botsford et al. [34] reported 36% HM (MOM and donor) grew Gram-negative bacteria after continuous tube feeding to preterm infants, and found that this was associated with feed intolerance and suspected sepsis.

In Australia, all donor milk is screened before and after pasteurization, and all donor milk is pasteurized. From the past 4 years of operating the PREM Bank, we do know that rigorous bacterial screening of donor milk will result in approximately 26% pasteurized milk being discarded (fig. 1).

This rigorous bacterial screening regime has shown that bacterial content of donated milk varies greatly between donors and even between individual donations by the same donor. Most of the donor milk cultured and pasteurized, and on occasion discarded as 'unsafe' by the PREM Bank, has been donated by other mothers of preterm infants in our unit and, as such, has been fed raw to their own infant apparently without incident.

The issue of culturing milk after pasteurization is most controversial. The PREM Bank has processed 1,919 batches of donor milk since establishment in 2006. Every batch has been cultured before and after pasteurization and, of these, 43 showed bacterial growth after pasteurization. Twenty-six of these

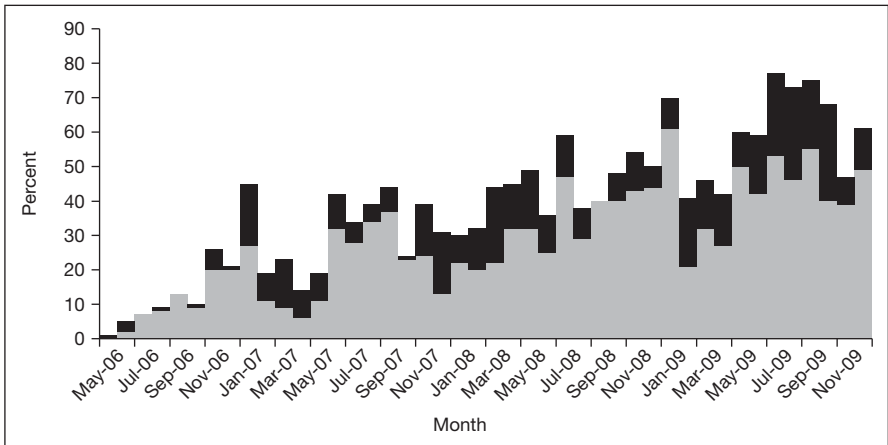


Fig. 1. Volume of PDHM rejected (black) due to bacterial quality in the PREM Bank.

43 passed the prepasteurization bacterial screen. *Bacillus* species grew in 32 of 43 postpasteurization cultures, including *Bacillus cereus*, a known food-borne pathogen. Postpasteurization obviously precludes use, and these batches are discarded. The germination of the vegetative spores of *B. cereus* is a well-documented consequence of heat treatment during food production [35, 36]. Only 7 of the 32 samples that grew *Bacillus* after pasteurization grew *Bacillus* before pasteurization. Without routine postpasteurization culture, one batch showing growth of a known food-borne pathogen would be released every 66 batches. At the PREM Bank, this would occur once every 5 weeks of processing. A similar issue with *Bacillus* culture after pasteurization of HM has recently been reported from the Austin HMB in Texas, USA [37]. We recommend post-pasteurization cultures for all donor milk dispensed to neonates.

Due to the risk of bacteria contamination and associated heat-stable toxins, all HMBs should consider implementing strict screening standards. In the case of the PREM Bank, the decision was driven by the risk assessment required during HACCP development. Although current evidence would suggest a low likelihood of bacteria that had been present in donor milk prior to pasteurization causing a clinical issue for a recipient of donor milk, the extreme vulnerability of our recipients currently dictates this cautious approach to bacterial screening. To ensure the viability of HM banking and its ability to operate in the most efficient manner possible, revision of bacterial screening of milk remains an issue requiring further research. The PREM Bank is currently developing real-time polymerase chain reaction method for the specific and rapid detection and quantification of bacteria and pathogens to allow milk

which is heavily contaminated or contains pathogens to be identified and discarded prior to pasteurization.

Alternative approaches are used internationally to increase efficiency and reduce cost while maintaining acceptable safety. In some countries, milk is streamed to be fed raw or pasteurized to very preterm and term infants based on level of contamination and risk to patient. For example, in Germany, donor milk with $<10^3$ CFU/ml is used for feeding infants $<1,500$ g either raw or pasteurized, whereas milk containing 10^4 – 10^5 CFU/ml is analyzed for pathogens and pasteurized for feeding older babies if pathogens <104 CFU/ml [38]. This appears to be a pragmatic approach to minimize the waste of donations, but in the context of evidence-based practice, may be difficult to implement in some countries.

Most HMBs pasteurize donor milk at 62.5°C for 30 min to eliminate bacteria and viruses. However, raw milk is used for preterm infants in some countries: raw donor milk has been used for feeding preterm infants for many years in Norway where donors are screened for HIV, hepatitis B and C, HTLV 1 and 2, and CMV, and the milk screened to ensure that it is free of pathogens and has low bacterial counts [39]; in Sweden, raw donor milk is used in 5 of the 27 HMBs for preterm infants [32].

Nutritional Adequacy and Variability of Pasteurized Donor Human Milk

In 1980, Björkstén et al. [40] demonstrated that pasteurization reduced the bioactivity of breast milk. In our HMB, the retention of IgA, lactoferrin and lysozyme after classical pasteurization at 62.5°C was 72.3 ± 3.6 , 21.8 ± 3.3 and $39.4 \pm 11.5\%$ ($n = 22$) [41]. It is possible to optimize the pasteurization temperature and improve pasteurization design to improve the quality of PDHM; for example, pasteurization at 57°C for 30 min retains 90% bioactivity and removes 99.9% of bacteria [41]. Bile salt-stimulated lipase is also inactivated by classical pasteurization, which will also remove the filaments from the HM fat globule [42]. These factors contribute to reduced fat absorption from PDHM vs. raw milk [43, 44].

The composition of donor milk is highly variable with mean (coefficient of variation) values for fat, protein and lactose being 4.16 (21%) g/100 ml, 1.35 (24%) g/100 ml and 6.71 (9%) g/100 ml, respectively ($n = 50$, PREM Bank). Most very preterm infants being fed HM are supplemented with HM fortifiers containing protein, calories and minerals. PREM Bank measures the nutritional composition of donor milk, and the data are used for nutritional audits and occasionally for individualized fortification. Standardization of nutritional product from HMB is possible but rarely practiced [45]. Infrared spectroscopy is a useful analytical method for determining the macronutrient content of HM, and recently the mid-infrared HM analyzer (Miris AB, Uppsala, Sweden)

requiring 2- to 3-ml milk samples has been validated for human fresh and frozen milk and for pasteurized HM [46].

The Influence of Establishing a Human Milk Bank on Breastfeeding Rates in the NICU

It is important to confirm that PDHM is replacing formula not MOM in the NICU. We performed an audit of breastfeeding rates at discharge home in infants born <30 weeks gestation and surviving >3 weeks. In the 6 months before we established our HMB, 45/74 (61%) of very preterm infants were breastfed on discharge vs. 60/80 (75%) in the 6-month period one year after the establishment of our HMB. 15% of very preterm infants received formula as their first feed before the HMB opened vs. none after. Of infants who receive some PDHM, 60% go home breastfeeding. In our NICU, consent for feeding PDHM is obtained by the lactation consultants who also support the mother to express her own milk. Increased awareness of the benefits of HM may be a contributing factor to the increased breastfeeding rates of mothers of preterm infants discharged from the NICU after establishment of an HMB [47].

Cost Evaluation

There have been previous attempts to compare the cost of operating an HMB with the potential cost savings derived from the reduction in incidence of NEC in NICU [48, 49]. In countries where the background rates of NEC are low and hospital costs differ, it may be difficult to transpose these cost/benefit models. However, simple comparisons can be made. In the US, private non-profit milk banks charge USD 3.00 per 30 ml of PDHM; this charge covers the cost of donor screening, milk processing and transport. This equates to approximately AUD 120 per liter of donor milk. Following our NICU standard feeding regime for a hypothetical 24-week CGA infant fed exclusively donor milk, we would expect to require 10 l of donor milk until discharge. Thus we could attribute a cost of AUD 1,200 to provide donor milk for this hypothetical infant. This cost equates to less than a single day's care in our NICU. Given that many of the complications attributed to artificial formula use may increase the complexity of care and length of stay, providing PDHM needs only to prevent a few of these complications to recoup the investment many times over.

The Way Forward

Standard pasteurization at 62.5°C reduces bioactivity of immune factors in HM by about 50%. At least 57°C is required to destroy bacterial cell wall,

and temperatures above this damage proteins. BSSL is destroyed at 45–55°C. Alternative technologies for treating HM need to be developed to better retain bioactivity. We are assessing new methods developed by the food and dairy industry. Interestingly, these technologies are used to improve taste and smell of dairy products, not bioactivity. Ultrasonic pasteurization (22–100 kHz) induces inertial cavitation and results in the formation of microscopic bubbles which rapidly collapse producing shock waves and localized heating. These mechanical forces disrupt cellular membranes leading to cell lysis. We have shown that combining ultrasound with low heat (45°C) will result in a significant increase in bacterial inactivation, especially of *Staphylococcus epidermidis* which has some resistance to cavitation. Thermo-ultrasonic pasteurization reduces bacterial contamination while retaining secretory IgA, lactoferrin, lysozyme and BSSL at 90, 78, 80 and 45%, respectively. In addition, this technology homogenizes HM, which will prevent fat separation and loss of energy during tube feeding [50]. Further improvements are required to better retain bioactivity especially of BSSL. We are currently developing methods based on ultraviolet light. The preliminary findings are encouraging and may result in a safe high-quality product to be trialed as a priority in the care of preterm infants.

HMBs are most successful when developed as part of a package to promote breastfeeding, and this is likely to provide the way forward for the further development and regulation of HMB in Australia. The establishment of the PREM Bank was only possible because of generous financial contributions from the community and, unsolicited donations of breast milk. The ongoing costs are incorporated into the budgets of the NICU and pathology department of our hospital, the only tertiary perinatal centre in WA. The government has commended the contribution of PREM Bank to the health of the community by presenting prestigious State and Health Department awards in our first few years of operation. Networks of HMB have been established in countries such as Brazil and Sweden with some coordination of activities. In our region of the world, PREM Bank is supporting the development of milk banks in Melbourne, Sydney, Brisbane, New Zealand and the Philippines (with UNICEF). Networks facilitate quality control and training, encourage collaboration and contribute to maintaining standards and reducing costs. A reference laboratory within each region or country could monitor compliance with safety guidelines, liaise with Government and coordinate educational activities. We have become the reference laboratory for our region, and the lessons that we have learnt and the protocols that we have developed are readily transmissible to other units.

Collaboration between HMBs and between networks will lead to data collections and clinical studies which will provide evidence for refining and improving the process of HM banking and ultimately clinical outcomes related to feeding preterm and sick infants.

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Discussion

Dr. Mohanty: What would be the cost of decontaminating 1 liter of bank milk?

Dr. Simmer: In American figures it is USD 120, but we can't actually cost it in Australia because it's just incorporated in our unit, and the nurses and the doctors and the equipment are all part of it. But the actual pasteurizer, there are cheaper versions being developed. We visited with the WHO the milk banks in the Philippines, and they just make a very simple water bath, so the standards and the risk-benefit ratio are different in different parts of the world.

Dr. Mohanty: Have you ever detected HIV virus in the milk?

Dr. Simmer: We have never found an HIV-positive mother on screening donors. But we wouldn't take the milk from an HIV-positive mother. CMV is very common in Australia, but that is destroyed by pasteurization.

Dr. Gottrand: Another use of human milk from a milk bank is for an infant with the short bowel syndrome, but the data supporting that this milk helps intestinal adaptation are insufficient. Could you comment on this use, and do you have any experience?

Dr. Simmer: At our Children's Hospital NICU, where babies with short gut syndrome go, we do use human milk. There was at first a little bit of resistance, clinicians wanting to use the elemental formula, but human milk, particularly when you are fed small quantities, is better.

Dr. Lack: You have shown some quite convincing data about protection of breast milk in the very sick newborn, but the question remains, these protective effects are they due to protective beneficial effects of the breast milk or is it cow's milk protein, for example, that may lead to a cellular form of allergy or reaction in NEC? In some of these other conditions, you always have to question is it the beneficial effects of the milk that are protecting you or is it something negative in cow's milk, it's constituents or protein? If it's the latter, then trying to recreate the best form of human breast milk might not be the best approach, and I wondered are there any trials for example trying to prevent NEC with extensively hydrolyzed milk formula or elemental amino acid-based formulas?

Dr. Simmer: Most of the babies do get a bovine protein fortifier anyway, so I think the human milk is protective. There are a lot of good things in human milk. A long time ago, elemental formula was associated with NEC compared with normal formula because it has a high osmolality, and also the elemental formulas are made for GIT pediatric patients and often have long-chain fats, elemental formulas are not really made for preterm infants. The preterm formulas are pretty good, but not as good as human milk. Why would you use another formula as the preterm formula is made perfectly for a preterm infant?

Dr. Lack: So, an elemental formula could be presumably adapted to make it more suitable if it was something about milk protein.

Dr. Simmer: Yes it could be, and you can even buy preterm formulas with hydrolyzed protein if that's what you want. Most of us are a bit nervous about it because there have been trials reporting poor growth of preterm infants fed hydrolyzed formula.

Dr. Saavedra: I have a comment relative to elemental or amino acid-based formulas for premature infants. We have been dealing with short bowel patients, and we started using amino acid formulas to see if we could get them off parenteral nutrition faster than with either hydrolyzed or intact protein formulas. There are no good control studies in children with post-NEC short bowel syndrome, but even though it appears we could get them off parenteral nutrition faster, these kids who are now 10–12 years old, they can't get even close to any whole protein because they are extremely allergic as opposed to children who we had on hydrolysates who are tolerating protein much better. We were actually tolerizing these babies by using hydrolyzed or intact proteins, which we could not do if we just used amino acid for a long period of time.

Dr. Jones: I was intrigued by your comment that there was bacterial contamination in normal breast milk in mothers and that you should pasteurize it. That goes to the whole hygiene hypothesis argument that doesn't seem to do healthy children any harm. My question is, is pasteurized and homogenized bank milk better than untouched bank milk?

Dr. Simmer: One of my colleagues has moved to head a unit where they pasteurize the milk of CMV-positive mums (mums' own milk). I think that this protocol could well cause more harm than good. However, our milk rooms in our NICUs have been run like a kitchen and not to the standard of a human milk bank. I think we could improve our food handling standards in the hospital milk room.

Dr. Stettler: A few years ago, there was a paper from Germany that showed that children who had been fed bank breast milk from diabetic mothers were more likely to become overweight or obese than those who had been fed breast milk from non-diabetic mothers [1]. I would like to know if you are familiar with that study, if you followed that literature and whether that has an implication on your practice.

Dr. Simmer: I am not familiar with the paper, and it's not a question that we asked donors. Women with diabetes do have more trouble establishing lactation, than non-diabetic women, so they would be infrequent donors.

Dr. Harding: You are using banked milk and adding a bovine fortifier, but there are clearly possibilities for human milk fortifier. Where do you see that going?

Dr. Simmer: Dr. Van Goudoever will talk about this too, but we have used the ultrafiltration method and you need a lot of human milk. You also have to pasteurize it and protein quality may be reduced. I really think we have to improve pasteurization technology before we move onto further processing human milk. Dr. Van Goudoever?

Dr. van Goudoever: It's the same answer; we are on the edge of basically of producing our own human milk-based fortifier. Active freeze drying is the process we are doing, and we do it on pasteurized milk. But it's complicated. Like Dr. Simmer says, you have to add all kinds of vitamins, minerals, so as a neonatologist you are trying to mimic Nestlé so to say, and that's hard.

Dr. Simmer: And we don't have any evidence to support freeze-drying milk. The actual bovine fortifiers are pretty good, and we'd need to do the trials human versus bovine. The bovine fortifier is going to be a lot cheaper than the human milk one.

Dr. van Goudoever: So, we agree on having more studies.

Dr. Simmer: On human milk fortifier, yes. I'd find it hard to randomize a thousand babies to formula if you had human milk there, but it's not impossible.

Dr. Harding: It will also be interesting to debate what the outcome measure should be for such trial.

Dr. Were: In areas where we do not have pasteurizers and we also do not have fortifiers freely available because of cost, how long would you be able to store mothers' own milk so that we can give the milk she lactated in the first 3 weeks to the baby for the subsequent 2 weeks? Do you have any information about that?

Dr. Simmer: In the freezer, 3 months.

Dr. Nagesh: We have a problem regarding the cost of running a human milk banking facility. A lot of units in India use single donor unpasteurized breast milk for a single baby after taking informed consent. What are your thoughts on this?

Dr. Simmer: I am not opposed to that. I think as a director of a unit I wouldn't encourage it, and I don't think our government would be particularly happy. But on a one-on-one basis, if both parties are happy with it, I would be comfortable. Certainly in our aboriginal community that happens quite a lot. So, officially we wouldn't condone it, but on an individual basis I would be comfortable with it.

Dr. Nagesh: But is pasteurization not required then?

Dr. Simmer: No, you have got one donor to one baby, you don't have to pasteurize that milk as long as the recipient mum is aware of the risk, even though it's low.

Dr. Haschke: One comment on the cost structure of donor's milk. You were saying that in your unit is grossly subsidized and you cannot really estimate how much it costs, considering the whole logistic procedure. You mentioned the figure of USD 120 per liter in the US. We visited the milk bank of the University of Iowa and tried a cost analysis, where we assumed that the university wouldn't subsidize everything (e.g. students collect the milk, the University provides the car, housing, etc.). Our estimates were in the range of USD 300–500 per liter. This implies that the donating mothers are not getting any money. I find it somehow unethical to sell human milk protein and not give the mothers anything who donate the milk. What is your position on commercial human milk protein?

Dr. Simmer: I am uncomfortable with mums donating milk and a company making money out of it. It's not the way most milk banks work around the world, so I wouldn't be that happy with it. The cost of handling donor milk is just absorbed into the running costs. We are never going to get pasteurized donor milk as cheap as formula. We do not plan to sell it to term babies. When you compare the costs with how extremely expensive intensive care is for those little preemies who are getting NEC, then I think the cost of HMB becomes acceptable.

Dr. Haschke: I agree with you. One thing which should be done is a health economic analysis based on a real simulation of conditions which apply. Even if the cost per liter of milk comes out higher, there could be a benefit for the population.

Dr. Simmer: I have been looking for PhD students to do just that. It would be good information to have.

Dr. Lack: You mentioned about restricting this to the very sick, but given that a lot of the benefits of breastfeeding have been demonstrated in the well baby and if you believe the data on prevention of atopy or IQ data, and we don't know how much breast milk is necessary to produce these benefits, would there be an argument made to extend this sort of banking delivery, and would there be the capacity to deliver it?

Dr. Simmer: We have to practice evidence-based medicine. Personally I am not that convinced about the atopy side of things and breast milk. I think there are many advantages of breast milk, I am not sure about reducing asthma. If we stay evidence based, it's the preemies who benefit, and they are in-patients. The whole thing of becoming a commercial company and selling milk is just not where most milk banks want to go. We started feeding pasteurised donor milk to patients less than 32 week gestations when we didn't have enough mothers' own milk. There were more patients than milk. Those at the highest NEC risk were prioritized. But now that we have got a bigger pasteurizer, we are liberalizing use to 33–34 weekers because they are still preemies and they are still in our hospital. So, we are using it more but only for in-patients, including the short gut type babies, who might be 3 or 4 months postterm but they are still in hospital.

Dr. van Goudoever: How are parents responding when you say you are 26 weeks of gestational age and then you go up to 32 weeks and then stop?

Dr. Simmer: We tell them at the beginning, that we have only got so much and at 34 weeks it will be stopped; If the clinician thinks that baby is particularly small and had a lot of problems, you are allowed to have donor milk for longer.

Dr. Goqwana: Are we right and are we not creating some problems when we say it's a milk bank? And a second question: If women are on medication for whatever reason, and that medication is secreted into the breast milk, do you accept the milk?

Dr. Simmer: I know, usually when we talk about banks, we think money, but there are also blood banks, and human tissue banks. There are screening questionnaires for the medications that the mums are on, and we use Tom Hale's book. There are very few medications really that you have to refuse the milk, but we look at how much is secreted in the milk and the category of drug. We check all that.

Dr. Stathatos: Are there any collection standards to decrease the possibility of contamination with bacteria?

Dr. Simmer: There is Medela produces a microwavable bag for collecting milk. We have done a study but I haven't got the data with me. On preliminary look, I don't think it made much difference. It would be a good area to study. It's not just skin contamination, there is also subclinical mastitis in mums who are expressing. Bacterial contamination varies from week to week, so you can't just test the mum's milk at 10 days and say, 'that's clear, we won't test it anymore'; you have to keep testing it.

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Short- and Long-Term Effects of Probiotics Administered Early in Life

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Abstract

The concept of manipulating the gut microbiota through the administration of probiotics during early life in order to reduce the risk of and prevent or treat diseases, including those that manifest in later life, is appealing. However, a cautious approach is needed, and the long-term consequences of such administration should be carefully evaluated. Concerns related to the early administration of probiotics include timing, i.e. the administration often begins in early infancy, sometimes at birth, when gut microbiota is not fully established, and duration, i.e. the daily administration of such products is prolonged (several weeks or months). In the case of non-breastfed infants, delivery may be in the form of a specific matrix (infant formula) that could be the only source of feeding of an infant over a prolonged period. Finally, the fact that beneficial as well as some detrimental effects are seen years after administration of probiotics during the first months of life raises concern that other long-term effects such as immunosuppression in later life may also occur. Currently, while some promising data exist, there are still more questions than answers. However, rapid progress in this area of research is expected and no doubt will bring about a number of exciting findings.

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Introduction

Interest of parents and health care providers with regard to infant nutrition is shifting towards health benefits beyond the provision of nutritional requirements. Emerging evidence suggests that the microbiota disturbances during early life may have consequences extending into adulthood. The pathogenesis of such diseases as asthma, allergy, atopy, type 1 diabetes, and inflammatory bowel disease has been linked to abnormal intestinal colonization. This has led to an interest in the development of strategies aimed at manipulating

the composition and metabolic activity of the gut microbiota, including the administration of probiotics or prebiotics or a combination of both (synbiotics) during early life. These products are currently gaining worldwide popularity and are increasingly being used in the pediatric population, including very young infants, despite some reservations regarding their efficacy and safety in this vulnerable population.

There are a number of issues and concerns related to the administration of probiotics, defined as microbial food supplements which, when administered in adequate amounts, have a beneficial effect on the host [1], early in life. First, the timing, i.e. the administration often begins in early infancy, sometimes at birth. Thus, the onset of administration is at a time when the gut microbiota is not fully established, and factors that influence microbiota may potentially permanently affect the development of the ecosystem. Second, the duration, i.e. the daily administration of such products is over a long time (several weeks or months). Third, in the case of non-breastfed infants, delivery may be in the form of a specific matrix (infant formula) that could be the only source of feeding of an infant over a prolonged period of time. This is in contrast to older children and adults in whom consumption of a probiotic product constitutes only a portion of their diets. Finally, evidence from at least one study with a long follow-up period documented that beneficial as well as unfavorable effects are seen years after the administration of probiotics during pregnancy and during the first months of life [2, 3]. Such an observation raises concern that other long-term effects, such as immunosuppression, may also occur in later life.

In this paper, the literature concerning the short- and long-term health effects of administering probiotics during early postnatal life, i.e. during the first weeks/months of life, both to preterm and term infants is summarized. For this purpose, the MEDLINE and the Cochrane Library databases were searched in September 2010. Priority was given to randomized controlled trials (RCTs) or their systematic reviews or meta-analyses. Further references were identified from the original articles and recent review articles. Evidence-based clinical practice guidelines developed by respected scientific societies or expert groups were also considered. The primary interest was in clinically relevant, short-term and long-term efficacy outcomes, such as those related to a reduced risk of disease, as well as in outcomes related to safety. Studies reporting the effects of probiotics administered beyond early infancy, whether for prophylactic or therapeutic reasons, have been discussed in detail elsewhere and are not reviewed here.

Probiotics for Preterm Infants

The rationale for probiotic supplementation of preterm infants is based on data demonstrating differences in the establishment of the intestinal

microbiota in preterm infants. Compared with healthy, full-term infants, the intestinal microbiota in preterm infants features a low number of species, and there is significantly delayed colonization with anaerobes, especially bifidobacteria [4–6]. Additionally, preterm infants are often cared for in intensive care units and receive broad-spectrum antibiotics, which further contributes to differences in colonization patterns. The possible consequences of abnormal patterns of colonization in preterm infants to health are not known. However, it has been speculated that they may contribute to increased susceptibility to infections and the pathogenesis of necrotizing enterocolitis (NEC). The latter is one of the most serious, life-threatening, gastrointestinal diseases, and it is characterized by various degrees of mucosal or transmural necrosis of the intestine. The incidence of NEC in infants is 5–10% [7]. The highest incidence is reported in infants with birthweights below 1,000 g, and the incidence decreases with increasing birthweights [8]. The exact cause of NEC remains unclear. However, in addition to prematurity, factors such as formula feeding, intestinal hypoxia-ischemia, and colonization with pathogenic microbiota are considered to play a role in the pathogenesis of NEC [9]. While clearly the most effective strategy for preventing NEC is feeding with human milk, it has also been suggested that the enteral administration of probiotics to preterm newborns could prevent infections, prevent NEC, and reduce the use of antibiotics [10].

A number of systematic reviews, with or without a meta-analysis, have reviewed data on the effects of the enteral administration of probiotics on the risks of NEC and mortality in preterm infants [11–14]. Among them, the most recent is the updated meta-analysis by Deshpande et al. [14] (search date: March 2009), which identified 11 RCTs, including 4 recent trials, and involved 2,176 preterm infants. Compared with the control group, preterm neonates in the probiotic group had a reduced risk of NEC (relative risk, RR, 0.35, 95% confidence interval, CI, 0.23–0.55) and all-cause mortality (RR 0.42, 95% CI 0.29–0.62), but there was no difference between groups in the risk of sepsis (RR 0.98, 95% CI 0.81–1.18). Heterogeneity between trials was low ($I^2 = 0\%$), suggesting that the benefit appears to be a true class effect despite known differences between individual probiotic microorganisms.

From a methodological point of view, this is a high-quality meta-analysis the results of which should be reliable and valid. The major concern with regard to this meta-analysis, as with many other meta-analyses in the area of probiotics, is whether it is appropriate to pool data on different microorganisms. Arguments for and against pooling data on different probiotics are presented in table 1 [15].

The findings of this meta-analysis have potentially important clinical and public health implications, and thus should be taken into careful consideration. Should this meta-analysis alter our practice? Recently, the Committee on Nutrition (CoN) of the European Society for Paediatric Gastroenterology,

Table 1. Arguments for and against pooling data on different probiotics [15]

Arguments for	Arguments against
Allows one to: • Increase sample size and power • Establish whether there is evidence of an effect • Determine the direction of the effect • Determine the size of the effect (and the 95% CI around the effect) • Assess the consistency of the effect across studies • Identify the most promising probiotic(s) and decide whether further research on these probiotics is substantiated	• Probiotic supplementation is not a homogeneous intervention • Pooling data from different genera, species, strains, and doses of probiotics obtained in different populations, presumably with variations in their native intestinal microbiota, may result in misleading conclusions • The risk is that the results could be erroneously extrapolated to other probiotics or other patient groups

Hepatology and Nutrition (ESPGHAN) concluded that the presently available data do not permit recommending the routine use of prebiotics or probiotics as food supplements in preterm infants to prevent NEC. The Committee also recommended that each probiotic strain and potential combinations need to be characterized separately for each product [16]. This position does not mean that the use of probiotics for preventing NEC should be totally discarded. Rather, in settings in which the incidence of NEC is high, one may consider the use of probiotics – single or in combination. However, care should be given to choose those that are the best studied, with the highest effect size, and the best safety profile [17]. In this respect, figure 1 depicts a meta-analysis of the effects of probiotics for preventing NEC, with subgroup analyses based on the type of probiotic administered. In addition to data presented in the meta-analysis by Deshpande et al. [14], it also includes the results of one of the most recently published trials [18]. It clearly shows that while evidence regarding the potential beneficial effects of probiotic supplementation in preterm infants is encouraging, not all probiotic microorganisms are equal in preventing NEC. Of note, with the exception of *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG, there are no data from more than single studies on given probiotic microorganism(s).

With regard to the long-term effects of probiotic administration to preterm infants, only one trial has addressed this issue thus far. Participants in an RCT designed to evaluate the efficacy of probiotics in preventing NEC were subsequently enrolled in a follow-up study. Oral administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* at 1 week after birth until discharge had no effect on growth, neurodevelopmental outcomes, and sensory outcomes at 3 years corrected age [19].

Overall, certain probiotics prevent NEC. Whether probiotic supplementation should become the standard of care is still under discussion. Before the routine use of probiotics in preterm infants, data regarding which products should be administered, at what dose, and for how long are needed. While awaiting new studies and consensus among specialists, it seems reasonable to discuss with parents current evidence regarding probiotics and let them decide whether the intervention might be beneficial.

Probiotics in Infant Formulae

In 2010, the ESPGHAN CoN systematically reviewed published evidence related to the safety and health effects of the administration of formulae supplemented with probiotics compared with unsupplemented formulae [20]. RCTs and quasi-RCTs (defined as studies in which the participants are allocated to different interventions using methods that are not random; for example, allocation may be based on the person's date of birth) or their systematic reviews/meta-analyses were considered for inclusion. Overall, 20 publications met the inclusion criteria. The methodological quality of the included RCTs varied. The most common problems were a lack of description of randomization procedures and/or allocation concealment. Many trials were underpowered for the assessment of specific outcomes. Only studies carried out in healthy term infants were included. The studies varied in the types of probiotics used. The most commonly studied probiotic was *Bifidobacterium animalis* ssp. *lactis* CNCMI-3446 (previously known as *Bifidobacterium bifidum* or *B. lactis* Bb-12); this probiotic was administered either alone, in combination with *Streptococcus thermophilus*, or in combination with *S. thermophilus* and *Lactobacillus helveticus*. Other probiotics studied were *Lactobacillus acidophilus johnsonii* La1, *Bifidobacterium longum* BL999 (BL999) and *Lactobacillus rhamnosus* LPR (LPR), LGG, *Lactobacillus reuteri* ATCC 55730, and *Lactobacillus salivarius* CECT5713. The doses of probiotics varied considerably. Also, the supplementation periods varied from 15 days to 8 months.

The Committee evaluated the following 2 ways of administering probiotic-supplemented formula: (1) administration that was started in infants ≤ 4 months of age (or ≤ 6 months of age provided they had not started complementary feeding) and continued for at least 2 weeks, and (2) administration of probiotic-supplemented infant or follow-on formula at any other age beyond early infancy and regardless of the duration of the intervention. Overall, the Committee concluded that for healthy infants, available scientific data suggest that the administration of probiotic-supplemented formula to infants does not raise safety concerns with regard to growth and short-term adverse effects. The administration of probiotic-supplemented infant formula during early life (≤ 4 months of age) does not result in any consistent clinical effects. The administration of a few probiotics supplemented to infant or

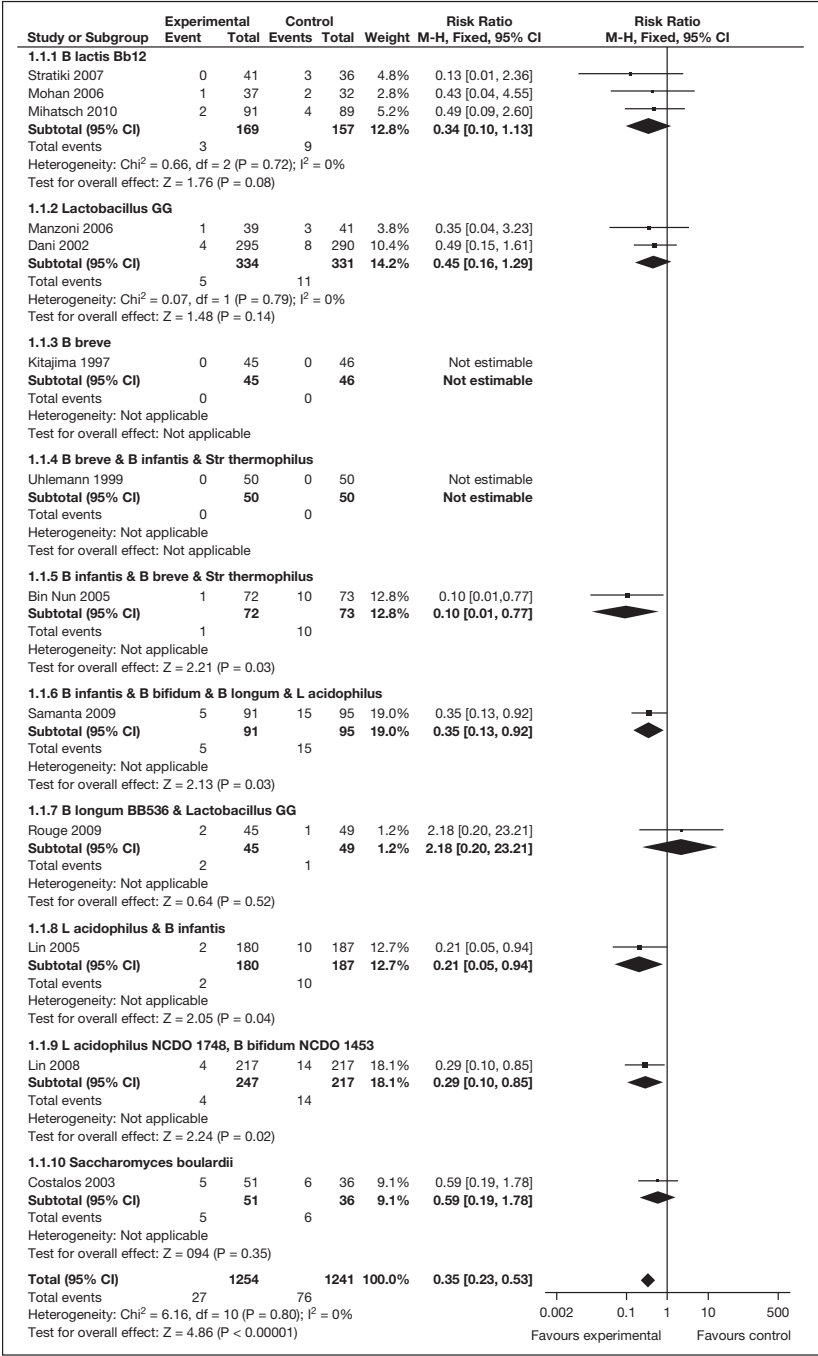


Fig. 1. Probiotics compared with control in the prevention of NEC in preterm infants – analysis based on probiotics strain(s) [18, 39–50].

follow-on formulae and given beyond early infancy may be associated with some clinical benefits, such as a reduction in the risk of antibiotic use, a lower frequency of colic and/or irritability, and a reduction in the risk of nonspecific gastrointestinal infections [particularly *B. lactis* Bb12 (single or in combination), 3 RCTs, $n = 302$, RR 0.54, 95% CI 0.4–0.8; fig. 2]; data related to other probiotics studied (i.e. *L. reuteri* ATCC 55730, *L. johnsonii* La1, *L. salivarius* CECT5713), whether positive or negative, are too limited to allow one to draw conclusions]. However, the available studies varied in methodological quality, the specific probiotics studied, the durations of the interventions, and the doses used. The Committee considered there is still too much uncertainty to draw reliable conclusions from the available data. The safety and clinical effects of one probiotic microorganism should not be extrapolated to other probiotic microorganism(s). In general, there is a lack of data on the long-term effects of the administration of formula supplemented with probiotics. The Committee stressed that such data would be of particular importance if the effects persist after the administration of the probiotic(s) has ceased.

Other Studies

A number of studies have evaluated the effects of probiotics introduced early in life but not in infant formula. Instead, they were administered in capsules, the contents of which were supplemented to breast milk and/or infant formula or given to an infant only. As these studies were not included in the systematic review carried out by the ESPGHAN CoN, they are briefly discussed below.

Primary Allergy Prevention

The rationale for using probiotics in the prevention of atopic disorders is based on several concepts. First, it has been suggested that improved hygiene and the reduced exposure of the immune system to the microbial stimulus early in childhood contribute to the rising number of allergic disorders worldwide [21]. Second, there are differences in the neonatal gut microflora that may either precede or coincide with the early development of atopy. Atopic subjects have more clostridia and tend to have fewer bifidobacteria species in their fecal microbiota than non-atopic subjects [22]. Finally, there is evidence suggesting a crucial role for a balanced commensal gut microflora in the maturation of the early immune system.

Clinical research on the effects of probiotic administration early in life on allergy prevention started only about a decade ago. So far, the evidence is conflicting. A recent Cochrane Review concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for the prevention of allergic diseases, including food hypersensitivity [23]. However, a meta-analysis by Lee et al. [24] demonstrated that the prenatal and/or postnatal administra-

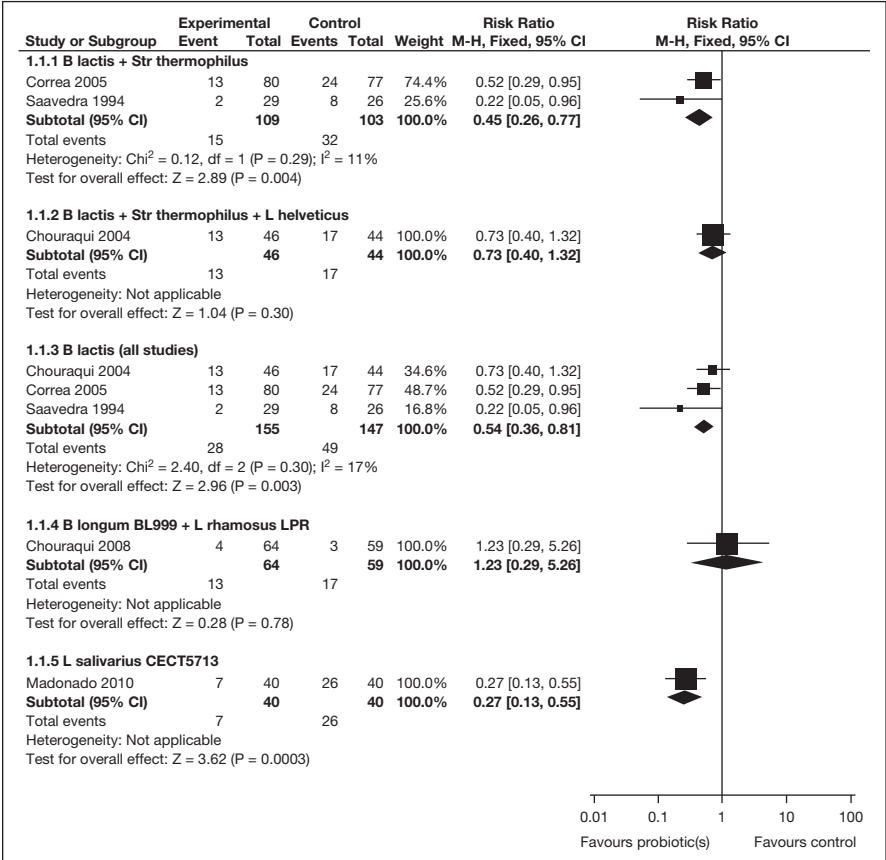


Fig. 2. Probiotic-supplemented formula for preventing gastrointestinal infections [51–55].

tion of probiotics to pregnant women and their infants resulted in a significant reduction in the incidence of atopic dermatitis in the infants. The authors of a third review also concluded that probiotics, especially *Lactobacillus* GG, seem to be effective in the prevention of atopic dermatitis [25].

As pointed our earlier, the merit of pooling data obtained on different probiotic strains, with no analyses based on probiotic strain(s), is questionable (table 1). The meta-analysis by Lee et al. [24] can be furthermore criticized for combining data from the same study population at different time intervals. Meta-analytical purists may also disagree with combining the results from 5 studies on probiotics with results from one study on synbiotics. Moreover, due to its subsequent publication, none of these meta-analyses included the recent negative data related to *Lactobacillus* GG [26]. This important study

was carried out based on a protocol almost identical to that used in an earlier positive study by Kalliomaki et al. [2, 3] with a long follow-up. The authors reported that supplementation with *Lactobacillus* GG during pregnancy and early infancy did not reduce either the incidence or severity of atopic dermatitis in affected children, but it increased the rate of recurrent episodes of wheezing bronchitis [26].

One additional RCT deserves discussion here. This is a double-blind RCT conducted in Finland in which 1,223 pregnant women carrying infants at high risk for the development of atopic disease were randomly assigned to receive either a combination of *L. rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS or placebo for 2–4 weeks before delivery. The infants received the same probiotic preparation plus galacto-oligosaccharides or placebo for 6 months after delivery. Clearly, simultaneous administration of probiotics and a prebiotic is not equal to the administration of probiotics only. However, the results of this study merit attention in the context of this chapter (i.e. early administration of the study products, large sample size, long follow-up). A significantly higher proportion of infants who received the probiotic/prebiotic preparation compared with placebo had a reduction in their risk of developing eczema (RR 0.81, 95% CI 0.66–0.99) and in their risk of developing atopic eczema (RR 0.70, 95% CI 0.51–0.96). However, there was no significant difference between groups in the cumulative incidence of all allergic diseases [27]. In a follow-up study that assessed outcomes at 5 years [28], no significant differences were found between the experimental and placebo groups in the frequencies of allergic disease, IgE-associated allergic disease, sensitization, eczema, atopic eczema, allergic rhinitis, and asthma. However, less IgE-associated allergic disease occurred in cesarean section-delivered children who received probiotics plus galacto-oligosaccharides compared with placebo (24.3 vs. 40.5%; $p = 0.035$). Thus, the preparation containing probiotics and prebiotics, at least as used in this study, did not result in an allergy-preventive effect that extended until the age of 5 years except in a subgroup of children delivered by cesarean section.

Overall, research in the area of prevention of allergic disorders through modification of intestinal microbiota is relatively new. Currently, there is insufficient evidence to recommend the addition of probiotics to infant feeds for the prevention of allergic disease. Long-term RCTs are necessary to establish whether manipulation of the gut microbiota can safely decrease the risk of allergic disease. If yes, we need to determine which microorganisms are suitable and in which type of population.

Prevention of Acute Infections

The immunomodulatory effects of probiotics have been studied extensively in both in vitro and animal models. The question that remains, however, is what is the clinical relevance of such immune modulation?

In a double-blind RCT, Finish investigators assigned 81 infants before the age of 2 months to receive probiotics (*Lactobacillus* GG and *B. lactis* Bb12) or placebo daily until the age of 12 months. The probiotics were administered in capsules, the contents of which were supplemented to infant formula. With regard to the primary outcomes, a group of 72 infants was available for the analysis. During the first 7 months of life, there was no significant difference between the probiotics and placebo groups in the incidence of gastrointestinal infections (RR 0.21, 95% CI 0.03–1.64) or respiratory infections (RR 0.89, 95% CI 0.67–1.18). However, there was a reduced incidence of acute otitis media in the probiotics group compared with the placebo group (RR 0.44, 95% CI 0.21–0.9). In addition, the investigators reported a significant reduction in the use of antibiotics in the group of infants whose formula was supplemented with *B. lactis* Bb12 and LGG compared with the control formula group (RR 0.52, 95% CI 0.29–0.92) [29].

Additional data come from the already described Finish study [27]. In 2008, these investigators reported data from a follow-up study in which a 2-year follow-up assessment was completed by 925 of 1,018 eligible infants [30]. During the 6-month intervention, there was no significant difference between the probiotic/prebiotic and placebo groups in the occurrence (at least once) of respiratory infections (66 vs. 68%), middle ear infections (15 vs. 19%), or gastroenteritis (13 vs. 14%). Fewer infants received antibiotics in the experimental group than in the placebo group, although the difference was of a borderline significance (23 vs. 28%; OR 0.74; 95% CI 0.55–1.00; $p = 0.049$). After the intervention, during the follow-up period (6–24 months), the total number of respiratory infections was significantly lower in the probiotic/prebiotic group than in the placebo group (geometric mean: 3.7 vs. 4.2 infections; ratio: 0.87; 95% CI 0.79–0.97; $p = 0.009$). The authors concluded that the study indicates that feeding probiotics and prebiotics to allergy-prone infants increases their resistance to respiratory infections during the first 2 years of life [30].

Overall, certain probiotics administered early in life have the potential to reduce the risk of various symptoms of respiratory tract infections. However, data are currently far too limited to distill any clinical recommendations.

Safety

Overall, probiotics are generally safe for use in otherwise healthy populations [31]. Still, if probiotics are to be given early in life, particularly if for a prolonged time, safety issues need special attention. As summarized earlier, the ESPGHAN CoN concluded that for healthy infants, available scientific data suggest that the administration of the probiotic-supplemented infant formulae studied so far does not raise safety concerns with regard to growth and short-term adverse effects [16]. However, the safety and clinical effects

of one probiotic microorganism should not be extrapolated to other probiotic microorganism(s). In the context of the discussion here, observations made by some investigators in studies with probiotic administration during early life, such as for example higher rates of some airways symptoms in the probiotic-supplemented group [2, 32, 33], merit further investigation.

Quality of Probiotic Products

Many clinicians have concerns regarding the reliability of some of the products currently on the market. Indeed, a number of studies have questioned the microbiological quality and labeling of many probiotic products [34–38]. Only some of them meet the definition of probiotics, i.e. contain viable, defined microorganisms in sufficient numbers. Products sold for medicinal purposes tend to be of better quality than probiotics used in dairy foods or probiotic supplements. Considering that the beneficial effects of probiotics seem to be strain specific and dose dependent, such results indicate the need for regulation concerning the labeling of probiotic products. Accurate labeling is essential for their proper use. Health care professionals and consumers should be aware of possible variations. Until issues are regulated, the only sound approach seems to be to choose probiotic-supplemented formula and/or a probiotic product from a recognized manufacturer who has a regulated quality control of factors including the composition and content of the probiotic bacteria. Ideally, the safety and effectiveness of the manufacturer's product should be confirmed in well-conducted RCTs.

Conclusions

The concept of manipulating the gut microbiota through the administration of probiotics during early life in order to reduce the risk of and prevent or treat diseases, including those that manifest in later life, is appealing. However, a cautious approach is needed, and long-term consequences of such administration should be carefully evaluated. As of now, there are still more questions than answers. However, rapid progress in this area of research is expected, and no doubt will bring about a number of exciting findings.

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Discussion

Dr. Villalpando: You addressed in your meta-analysis the total amount of the probiotics needed for preventive purposes. Does the dose of probiotics in supplemented milk make a difference to a preterm infant?

Dr. Szajewska: The problem with the meta-analysis is that all of the probiotics were pooled together, i.e., different probiotics, doses, durations and ways of administration, given with breast milk or with formula, etc. So, I don't have a clear answer to tell you regarding what is the total dose. In most of the studies, the dose was 10^8 to 10^9 CFU. It depends very much on the study.

Dr. Villalpando: This is a very important issue in relation to obesity. The Kalliomäki group worked with the cohort that was supplemented with probiotics early in life [1].

Dr. Szajewska: The intervention started 2 to 4 weeks before birth when probiotics were given to expecting mothers and then they were given 6 months after delivery either to breastfeeding mothers or their infants. But the children who were evaluated here were not the whole cohort, and there was no statistically significant difference in outcomes [2]. I showed it because it's something that is very often discussed. It's an interesting finding, but definitely I am not saying that we should jump to the conclusion that this particular probiotic, or any probiotic would really prevent obesity. I think it's much too early to jump to these kinds of conclusions.

Dr. Mohanty: We have an ongoing randomized controlled study in our hospital; 3 centers have been chosen under the funding of the American Health Department, and probiotics have been used in over 5,000 preterm newborn infants on days 1, 3, 5 and 7 in the hospital; then, they were followed in the health centers in the rural areas. I was one of the evaluators of that study. Although we have not made a final analysis yet, the interim analysis is very encouraging. In terms of reducing infection, it has a very good salutary effect. But what is interesting is, the skin is the largest organ in the body, why are we not trying something on the surface of the skin to prevent infection maybe in the extreme preterm babies?

Dr. Szajewska: That's an interesting concept. I don't really know whether it would be more effective or not; I simply don't know.

Dr. Mohanty: In Saudi Arabia in 1982, I tried administering probiotics using this method.

Dr. Szajewska: But my concern would be whether these probiotics could survive on the skin. I think there would be a number of issues regarding how to administer the probiotics, how to make sure that they would still be alive, and how to determine whether they need to be alive.

Dr. Mohanty: I used them just after delivery. But the mothers complained that I made their babies dirty and sticky, so I discontinued this.

Dr. Fasano: Soon after that, this meta-analysis was published in *Pediatrics*, and all the comments were associated with it; both the NIH and the FDA in the US took a very strong position. As a matter of fact, if you propose to use probiotics for improving health it's one thing, but if you propose to use probiotics with specific therapeutic indications, you leave the premises of food supplement and you come into drug development, meaning that you have to go through the same scrutiny as any drug, i.e. put together very complex and cumbersome package that's technically called IND, and then you have to go through safety and efficacy trials and so on and so forth. Interestingly enough, the NIH empowered National Center for Complementary and Alternative Medicine to be in charge of this kind of situation. The bottom line is that if you want to do any study, and it is NIH funded, there is no way that you can use any probiotic unless you address the question that you put on the table, no matter what kind of meta-analysis you go for. Not all probiotics are made equal. When penicillin was discovered, we thought we could treat any infection with it. Now we know too well that this is not the case and indiscriminate use of antibiotics can be detrimental. The same story here, probiotics probably will need to be customized to any specific intervention. So we came up with the argument that the meta-analysis for NEC, for example, is so strong to justify the use of probiotics in preterm infants. However, NIH and FDA claim that if you attempt to use probiotics for a specific indication, they need to be treated as drugs and, as such, you have to show composition, stability, if the content would change over time, the shelf life and so on. I think you were right when you said that evidence base is by no means something that needs to be disregarded; but the game with probiotics has been played in a very unusual way. We use them first and then we try to figure out how they really work, rather than the other way round.

Dr. Szajewska: I have to agree particularly with the latter comment. My position is that if we want to use probiotics as a medicine we should really do these kinds of studies. This is exactly my position.

Dr. Rings: I have a question concerning premature babies and NEC prevention. I would guess very young children with very low birthweight will profit most from probiotics, and my question is: are there sufficient data for this particular group, because they have the highest incidence of NEC? Are there enough data, to your opinion, to promote probiotics for this particular group if there are no restrictions in supplying them?

Dr. Szajewska: This issue has been raised by the authors of many meta-analyses, including the authors of the Cochrane review. There is not enough data with regard to extremely very low birth weight infants, those below 1000 g. As you said, this is a group that is particularly at risk for NEC. When I was showing one of the sub-group analyses that could be done, one option is to do it based on birth weight and gestational age. Definitely the risk is different depending on the birth weight and gestational age. I agree, not enough data.

Dr. Klish: I want to ask you a very specific question about safety which has been bothering me for the last few months since I was on a panel where this issue came

up. It has to do with *Lactobacillus reuteri* which is one of the probiotics that you discussed. *L. reuteri* produces reuterin, and reuterin at least theoretically can be a mucosal toxin. The question that came up was whether it is safe to use this particular probiotic in the first few days of an infant's life when the bacterial matrix is not well developed. We know that it is safe later in life, but is it safe in your opinion in the first few days of life?

Dr. Szajewska: The problem is we simply do not have enough evidence, even though nowadays this is probably one of the best-studied probiotics. My answer would be we don't have sufficient evidence to answer yes or no.

Dr. Klish: I think that is a problem because there may be attempts to use this probiotic in that way without any knowledge of its effect.

Dr. Guandalini: A short comment on one of the points that was raised on the very low birthweight and the use of probiotics to prevent NEC. Erika Isolauri's group have recently published their retrospective experience in Finland with a number of neonatal intensive care units that have been using LGG routinely in their infants [3].

They looked at it retrospectively, and documented that actually in some instances the use of LGG was detrimental: the prematures that received LGG had indeed a higher rate of NEC. However, if you look at the data, you would notice that all the numbers that lead to this total increase in prevalence of NEC in the probiotic-treated group refer to very low-birthweight babies. In fact, those who were 1,500 g or more had actually an equal or lower incidence of NEC. So, I believe it is fair to say that the jury is still out on this, but certainly the evidence of efficacy of probiotics in preventing NEC is quite strong. For this reason, it becomes imperative that well-controlled trials be performed in the USA, considering the reluctance that physicians in this country seem to show when it comes to accepting scientific evidence that is generated outside of the national boundaries.

Dr. Simmer: I feel I have to say something because the two meta-analyses in the *Lancet* and *Pediatrics* from people in my unit, in my team, their strong conclusion wasn't that there shouldn't be further trials, it was questioning the use of placebo in their trials. Since the paper in the *Lancet*, for 2 years we have done exactly what you have said, we have got every product available to us and every single one of them was insufficient, the whole thing was a disaster for 2 years. Now, we have located two, and even though the meta-analysis says not for placebo, until we use them in our 23, 24 weekers we are doing placebo but not on 4,000 infants to look at NEC, on 100 infants just to look at colonization. Future trials will be between different probiotics, between different doses, that sort of thing. So it was never that there was no need for further research, it was questioning the ethics of how long can we not give any probiotic to a premature baby with NEC.

Dr. Szajewska: Can I ask you a question? If the conclusion is that we should not do placebo-controlled trials with probiotics for preventing of NEC, which probiotic should we choose to administer to the control group? I have to say that I would have problems choosing the proper one. That's why I have some doubts about agreeing with this conclusion, I think we still need placebo-controlled trials. But if yes, which one of the probiotics to choose?

Dr. Simmer: The one that we've been using is Infloran and the other one is one from Japan, where in the Japanese literature there has been a lot of trials, but they are the only two. Even for Infloran it took a year to get the product that was stable, so there is a lot of laboratory work before you can give it to the baby.

Dr. Szajewska: With regard to Infloran, there are two studies that used different compositions of Infloran [4, 5]. Contradictory results from the studies on *Lactobacillus GG* from Finland [6] and from Germany [7] show how much we need repeat studies to confirm the results. With Infloran, whatever composition it was, it was a single study.

The results were not confirmed in another study. So, I would agree with the conclusion provided we have at least another such large trial. Then, it is OK to say that this is a standard for treatment now, and now let's do other studies comparing probiotics with one which really have been proven to be effective.

Dr. Saavedra: Maybe just a couple of comments that relate to what was said earlier on the regulatory side. Unfortunately, the way the law is written is that a specific product is classified depending on what you say about it, independent of what the product does. Ultimately, it is the claim that defines the product, not the effect. So if today, and I do advise a number of fellows and postdoctoral research students who are writing their applications to NIH or any other federal funding, if you say that this trial is designed to look at the use of a particular functional product, in this case let's say it is a probiotic, a particular species or strain, for the purposes of preventing diarrhea, you will need an IND number, you will need a drug number, your fellowship will end and you didn't start the study. However, if you make the same application with the assumption that what you intend to is to look at the frequency of loose stools in children over a period of time, you will not need an IND number because you are not preventing a disease. So from that point of view, some people get lucky, get their project through, some still have a big problem, and the other next level that they have to go through is their own IRBs in the university which have exactly the same issue.

Dr. Fasano: I have been tangled with two grants that were given and then stopped until I got the IND, so it is true that in the past you had the chance to twist the system. Not anymore in the sense that now they have a panel in which lawyers, scientists and policy makers are involved, funded by the NIH to rewrite the rules because we say this is not fair, you can't change the rules while you play the game and they did exactly that, and if you like it or not, that's the way it goes. And just as a corollary when you are discussing which probiotic you use, again it doesn't matter because it's not efficacy we are talking about here. First you need to show safety, and then you talk about efficacy.

Dr. Saavedra: It may be illustrating to the audience just to get an idea of what it took to get *B. lactis* strain BB12 approved by FDA here. It took approximately 7 years. The safety dossier is not the clinical trials. If the incidence of infection due to a bacterium is going to be one in a thousand, you would have to study about 500,000 children to even say it's going to happen. The safety dossier for an agency like the FDA is composed first of the bacterium description itself, what is the bacterium, what does it do, does it have toxins, is it heat stable, etc. In fact, the full genome of *B. lactis* was presented to the FDA before the FDA approved it. So, this is how long it took, but nevertheless this is what it ultimately takes to make these steps, that is the only way to do it obviously. My other comment has to do with the quality control of the product. It is absolutely true that the majority of supplements in this country are regulated very different than food. With the supplement industry there are no rules that unfortunately are enforced to the point that you can truly be sure, and that's the point of getting an adequate manufacturer that will give you quality control data through shelf life.

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Complementary Feeding and the Health and Development of the Infant over the Immediate Period of Infancy and Early Childhood

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New Findings from the Feeding Infants and Toddlers Study 2008

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Abstract

The purpose of this chapter is to describe the infant feeding practices among infants and toddlers (aged 0–24 months) and to describe food group consumption patterns of these infants and young children (0–48 months) participating in the 2008 Feeding Infants and Toddlers Study (FITS). The FITS 2008 is a cross-sectional survey of a national sample of US children (n = 3,273). Results indicate a longer duration of breastfeeding; however, 17% of infants received cow's milk before the recommended age of one year. Introduction of complementary foods also appears to be delayed until about 4–6 months. There was a decline in consumption of infant cereal after 8 months that may be contributing to iron deficiencies in the 9–11 months age group. Consumption of 100% juice (particularly among infants) and the daily consumption of desserts or candy, sweetened beverages (particularly among 12- to 20-month-olds), and salty snacks is lower than in the 2002 survey. Overall, 10–20 and 30% of children were not consuming any fruit or vegetable, respectively, in a given day. More preschoolers were drinking 2% milk than whole milk, but about one third were still drinking whole milk. Despite some of these positive changes, improvements in young children's diet still are needed.

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Introduction

Providing adequate early childhood nutrition is a major concern across the globe. Improper infant feeding, including issues related to breastfeeding, use of human milk substitutes and complementary feeding are linked both with overall inadequate nutrient and food consumption patterns and concerns related to energy imbalance and excessive weight gain [1]. For preschoolers

(2- to 4-year-olds), issues such as increased frequency of eating and increased portion sizes are linked with overall poor dietary patterns [1]. Limited information exists from large, national samples in the US that can contribute to our understanding of the parental behaviors potentially leading to early childhood obesity. The previous Feeding Infants and Toddlers Study (FITS), conducted in 2002 on a national random sample, was instrumental in documenting the feeding patterns of children from 4 to 24 months of age [2, 3]. These data provided incredible insights into some of the problems that may be contributing to the increased rates of obesity at an early age, such as the early introduction of foods during infancy that are high in fat, sugar and sodium reflective of diets found in older children and adults [2]. With the completion of yet another survey conducted in 2008, we have the opportunity to explore how food consumption patterns have changed over time. This newer survey includes children beyond the age of 2 years. Thus, the purpose of this paper is to describe the infant feeding practices in terms of breastfeeding and use of human milk substitutes as well as the introduction of complementary foods among infants and toddlers (aged 0–24 months) and to describe food group consumption patterns of these infants and young children (0–48 months) participating in FITS 2008.

Methods

Study Design and Response Rates

The FITS 2008 is a cross-sectional survey of a national random sample of US children from birth through 47 months of age similar to the initial survey conducted in 2002, but with an expanded age group [4]. The recruitment of subjects, the sampling frame, sample characteristics, and data collection and quality assurance procedures have been previously described in detail by Briefel et al. [5]. Among respondents who completed the recruitment interview, 78% completed a 24-hour dietary recall. The overall analytic response rate among those located with an eligible child was 47%. All instruments and procedures were reviewed and approved by Mathematica Policy Research's independent Institutional Review Board (Public/Private Ventures in Philadelphia, PA, USA).

Sample

The FITS 2008 sample size for the dietary analysis includes 3,273 children aged 0–48 months. The results presented in this article are divided primarily into four groups according to the following age ranges: infants aged 0–5.9 months (meaning from birth up to 6 months; $n = 382$), older infants aged 6–11.9 months ($n = 505$), toddlers aged 12–23.9 months ($n = 925$), and young children aged 24–47.9 months ($n = 1,461$). It is important to note that because of the variable feeding practices present in the younger age groups, data will often be mentioned in terms of nine smaller subgroups – infants aged 0–3.9 months ($n = 216$), 4–5.9 months ($n = 166$), 6–8.9 months ($n = 249$), and 9–11.9 months ($n = 256$); toddlers aged 12–14.9 months ($n = 243$), 15–17.9 months ($n = 251$), 18–20.9 months ($n = 219$), and 21–23.9 months ($n = 212$), and finally young children aged 24–47.9 months ($n = 1,461$). These separations serve to clarify nutritional differences inherent in breastfeeding rates over time and illuminate

certain findings within the survey data relevant to the increasing reliance upon other forms of food intake as the child develops during the first 4 years of life.

Statistical Analysis

All foods and beverages reported in the 24-hour dietary recalls were assigned by Mathematica nutrition researchers to food groups in a manner consistent with those used for the food group analysis in the 2002 FITS [2]. The 2002 food groups were updated and expanded, as needed, to incorporate new foods and beverages reported in the FITS 2008 and to address the research objectives on consumption of foods and food groups/subgroups.

We used the food group data to calculate the percentage of children who consumed specific foods or food groups at least once in a day. One-day estimates from 24-hour recalls for the purpose of estimating group means has been previously shown to be appropriate [6]. All reported foods and beverages are included in the estimates, regardless of the amount consumed. Estimates are based on foods as consumed, i.e. food mixtures, such as soups, pizza, or pasta-based dishes, are considered single items and were not broken down into their constituent ingredients. In this manner, the estimates of the percentages of infants and toddlers consuming vegetables and fruits should be considered as lower-bound estimates.

Sample weights were calculated to account for nonresponse and to weight the sample to known population demographic characteristics. All estimates (e.g. means, proportions) were calculated using the Statistical Analysis System (version 9.1.3, 2004, SAS Institute, Cary, NC, USA) and accounted for the weighting and design effects. Standard errors were calculated using SUDAAN (release 9, 2005, Research Triangle Institute, Research Triangle Park, NC, USA).

Results

Population Characteristics

About 56% of the dietary sample was non-Hispanic white, 14% non-Hispanic black, 21% Hispanic, and 8% other race/ethnicity. About one third (35%) of children were first born, 48% were in child care, and 30% were receiving benefits from the Special Supplemental Nutrition Program for Women, Infants, and Children. About half (51%) of the children's mothers worked outside the home, and 46% had a college degree or higher. About 13% of the sample had annual household incomes below USD 20,000 and 16% above USD 100,000. Additional information on sample characteristics is described elsewhere [5].

Infant Feeding Practices

In 2008, rates for infants and toddlers aged from 0 to 23.9 months being 'ever breastfed' were high ($78.5 \pm 1.3\%$, mean $\pm$ standard error). Figure 1 shows little difference between the three youngest age groups (0–5.9 months, 6–11.9 months, and 12–24 months) in terms of their having ever been breastfed, characterized by a range of percentages from 74.9 ± 4.2 to $83.8 \pm 3.6\%$ for children aged 6–8.9 and 21–23.9 months, respectively. The percentage of infants currently breastfeeding was highest for the 0–3.9 months age subgroup ($59.5 \pm 5.2\%$), while a considerably smaller percentage from the next

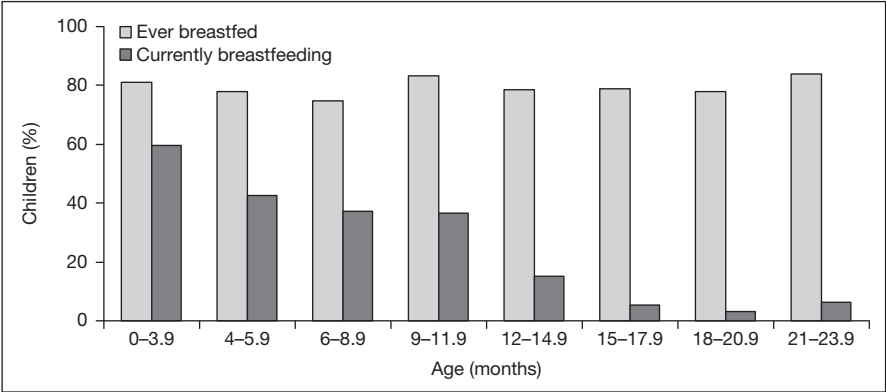


Fig. 1. Percentage of children ever breastfed and currently breastfeeding by age groupings in the first 2 years of life in the FITS 2008.

subgroup (4–5.9 months of age) were currently breastfeeding ($42.5 \pm 5.1\%$). Around the one-year age milestone, breastfeeding continues to decrease: $36.7 \pm 5.0\%$ of infants aged 9–11.9 months were breastfeeding, whereas only $15.1 \pm 3.1\%$ of 12- to 14.9-month-old children were doing so. A decrease in breastfeeding with increasing age was consistent throughout the older age subgroups, with rates of breastfeeding among toddlers aged 15–23.9 months in the single digits.

Figure 2 shows the percentage of infants and toddlers consuming different types of milk at least once a day, thus more than one type of milk can be consumed. The only age group for which breastfeeding was the leading milk source was the 0–3.9 months infant group; $57.5 \pm 5.3\%$ of these infants consumed breast milk on any given day, while $56.5 \pm 5.6\%$ consumed formula. For 4- to 5.9-month-olds, $42.2 \pm 5.1\%$ consumed breast milk on a given day and $65.3 \pm 5.0\%$ consumed formula. Cow’s milk is first seen among 6- to 8.9-month-olds, with 5.3% reporting consumption, and the proportion increases to 16.6% among the 9–11.9 months age group. As expected, breast milk and formula decrease as milk sources in the 2nd year of life, with cow’s milk serving as the primary form of milk for toddlers. Nearly 90% of toddlers aged 21–23.9 months consumed cow’s milk on a daily basis, whereas breast milk and formula were only consumed by 5.6 ± 2.3 and $1.3 \pm 0.8\%$ of children, respectively. Likewise, children aged from 24 to 47.9 months relied almost exclusively on cow’s milk. Of the different kinds of cow’s milk consumed by toddlers, the most common was whole milk (consumed by 59.8 ± 4.8 to $68.7 \pm 4.8\%$ of those toddlers aged 12–23.9 months). As these children aged, consumption of reduced fat forms of milk (including 1–2% fat and non-fat milks) became increasingly common; reduced fat milks were consumed daily by 14.1,

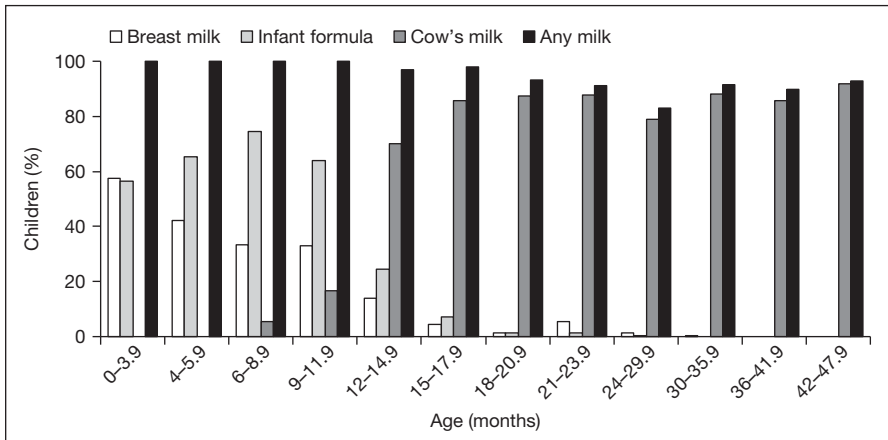


Fig. 2. Percentage of children consuming various forms of milk by age groupings in the first 4 years of life in the FITS 2008.

49.8 and 71.0% of children aged 12–14.9 months, 24–29.9 months and 36–41.9 months, respectively. There was an increase in the percentage of older toddlers (21–23.9 months old) consuming flavored milk on a daily basis, $11.2 \pm 4.4\%$, markedly up from $1.0 \pm 0.5\%$ among 18- to 20.9-month-old toddlers.

The use of complementary foods is shown across all age groups in figure 3. For infants aged 0–3.9 months, there was negligible consumption of complementary foods other than grains (10.9%, predominately in the form of infant cereal). Introduction of the other complementary foods took place to some degree at 4–5.9 months of age, with the majority (52.0%) of children in this age group consuming grains (fig. 3); specifically, 50.4% of children aged 4–5.9 months were consuming infant cereal within the grains food group (fig. 4). Infant cereal consumption peaked in the 6–8.9 months age group at a level of 79.1%, tailing off gradually to a level of 8.0% by 15–17.9 months. Referring again to figure 3, with increased age from 0 to 11.9 months, there was a consistent pattern of increase in the percentage of children consuming from each of the complementary food groups (grains, vegetables, fruits/juices, meats/fish/eggs/nuts, and sweets/sweetened beverages/salty snacks). In the 4–5.9 months age group, vegetables and fruits were being consumed by 25.9 and 21.8% of infants, respectively, with meats and sweets being consumed at frequencies below 5%. For infants 6–8.9 months of age, fruits, vegetables and meats had all three surged in importance to the complementary diet, with 62.8, 76.9 and 38.5% of infants consuming from these groups, respectively. By the last quarter of the first year of life, increases in the percentages of children experiencing intake of grains, vegetables and fruits stabilizes at high levels: 92.2, 72.3 and 89.8%, respectively. Protein and sweet intake contin-

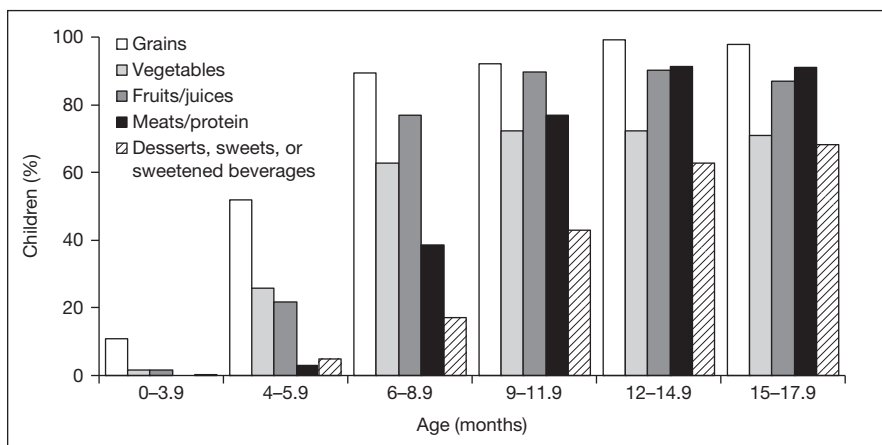


Fig. 3. Percentage of children consuming various complementary foods by age groupings in the first 18 months of life in the FITS 2008.

ued steady growth halfway into the 2nd year, when all complementary groups showed somewhat consistent consumption levels at or above 60%.

Grains

By the age of 6–8.9 months, nearly 90% of children consumed some grain product daily, and by the beginning of the 2nd year grain intake had plateaued at a level approaching 99% among 12- to 14.9-month-old toddlers (fig. 3). By 12–14.9 months, less than one quarter of children consumed infant cereals, down from $79.1 \pm 3.5\%$ at 6–8.9 months of age (fig. 4). During the span between 6–8.9 and 12–14.9 months, consumption of any non-infant cereal at least once per day increased from 7.7 ± 2.2 to $62.5 \pm 4.6\%$. At the end of the 2nd year, non-infant cereal intake had maintained a constant consumption level around 60% among toddlers. In the 4th year of life, negligible amounts of infant cereal was consumed, while a steady 50% of children consumed non-infant cereal, two thirds of which was whole grain (data not shown). Presweetened non-infant cereals were consumed in increasing amounts from age 12–14.9 months ($19.1 \pm 3.2\%$) to 21–23.9 months ($30.0 \pm 4.6\%$), whereas non-presweetened cereals decreased from 47.4 ± 4.9 to $30.0 \pm 4.9\%$ over the same age interval. For children of 2 and 3 years of age, these 30% intake levels of both presweetened and non-sweetened were maintained with little variation. Over the entire span from 12 to 47.9 months of age, whole-grain cereals were eaten far more commonly than non-whole grain (by a factor of 2 to 3) across all age groups in which non-infant cereals were consumed. Bread and rolls were consumed at least once a day by 2.6 and 14.3% of infants aged

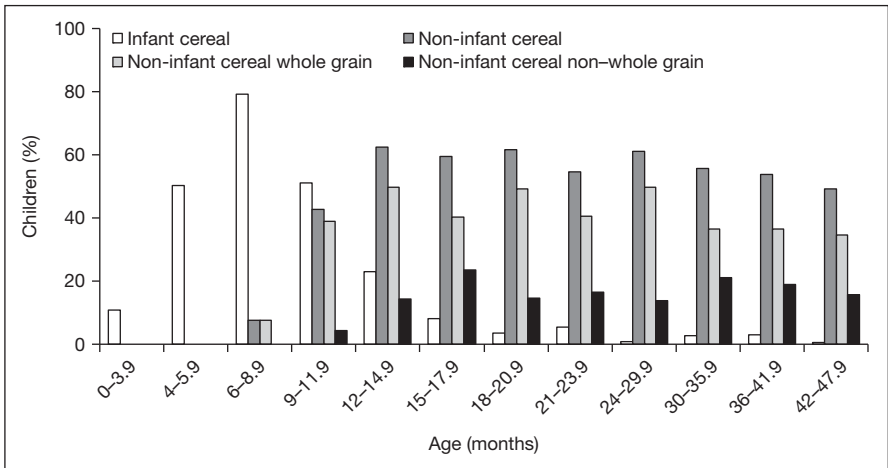


Fig. 4. Percentage of children consuming various forms of grain by age groupings in the first 4 years of life in the FITS 2008.

6–8.9 and 9–11.9 months of age and then remained at roughly 30% for all age groups thereafter. Other major sources of grain were: snack foods such as crackers, pretzels and rice cakes; rice and pasta; sandwiches, macaroni, pizza and mixed pastas (such as spaghetti and lasagna). Each of these food types was consumed by small percentages of infants younger than one year, but achieved higher and relatively stable levels of consumption among toddlers (data not shown).

Fruits and Juices

Fruits and fruit juices were introduced in many cases ($21.8 \pm 3.9\%$) to the diets of 4- to 5.9-month-old infants, and soon became commonplace (fig. 3 and 5), present in the diets of approximately 90% of toddlers between 12 and 23.9 months of age. After 9 months of age, 100% fruit juice contributes to approximately 50% or greater of total fruit intake (fig. 5). The intake of baby food fruit decreased by the middle of the 2nd year, from $50.2 \pm 5.4\%$ for infants aged 6–8.9 months to $15.9 \pm 3.7\%$ for toddlers 6 months older; in contrast, non-baby food fruit rose from 21.2 ± 5.2 to $68.8 \pm 4.4\%$ over the same 6-month interval. Non-baby food fruits began to be consumed by the majority ($51.9 \pm 5.0\%$) of children at age 9–11.9 months and became more common yet ($72.2 \pm 4.6\%$) as children aged to 24 months. Bananas were the primary source of fresh fruit through the end of the first year, at which point apples and grapes became more common in the diets of toddlers; applesauce and peaches served as the primary canned fruits consumed when children were no longer being fed baby food fruits.

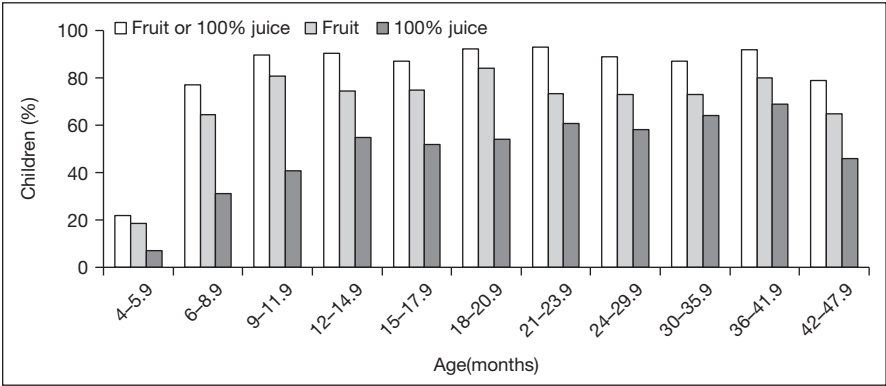


Fig. 5. Percentage of children consuming various fruits and fruit juices by age groupings in the first 4 years of life in the FITS 2008.

Vegetables

Vegetables were introduced to the diets of infants similarly to fruits, with a quarter of those aged 4–5.9 months consuming any vegetable at least once day (table 1). Unlike fruits, however, vegetables never attained the same high levels of intake throughout the later age groups, though they grew rapidly in importance to children’s diets between 4 and 11.9 months. Approximately 70% of older infants (over 6 months), toddlers and 2- and 3-year olds ate any vegetable at least once a day. Over half of infants consumed baby food vegetables at age 6–8.9 months, while just 15% of toddlers 6 months older did so. As baby food consumption decreased between these two age groups, cooked vegetable consumption increased by four times to 61%, at which point it remained relatively constant through the remainder of the second year of life. The proportion of children consuming dark green vegetables (such as broccoli, greens and lettuce), orange vegetables (such as carrots and sweet potatoes) and other vegetables such as mashed potatoes, corn, peas, onions and tomatoes) at least once a day varied across the ages as shown in table 1. The proportion consuming French fries was negligible at 6–8.9 months of age, increased to 6.3% at 9–11.9 and then remained in the range of 15–20% thereafter.

Meats, Fish, Eggs, and Nuts

Percentages of infants and toddlers consuming different types of protein sources are presented in table 2. While fruits and vegetables were first introduced to infants between 4 and 5.9 months of age, we see that meats and proteins were not introduced until slightly later, at 6–8.9 months, during which time nearly half of children consumed a protein source at least once a day,

Table 1. Percentage of children consuming different types of vegetables at least once in a day

	0-5 months		6-11 months		12-23 months			24-35 months			36-47 months	
	0-3.9	4-5.9	6-8.9	9-11.9	12-14.9	15-17.9	18-20.9	21-23.9	24-29.9	30-35.9	36-41.9	42-47.9
Any Vegetable	1.5 ± 0.9 ^a	25.9 ± 4.6	62.8 ± 5.3	72.3 ± 4.8	72.4 ± 4.7	70.8 ± 5.0	72.1 ± 4.1	68.1 ± 5.7	70.2 ± 4.0	71.8 ± 3.9	66.8 ± 3.9	69.8 ± 3.8
Baby food vegetables	1.1 ± 0.8 ^a	24.3 ± 4.6	51.3 ± 5.3	33.8 ± 4.3	15.1 ± 3.5	7.6 ± 2.3 ^a	2.7 ± 1.6	1.5 ± 1.0	0.2 ± 0.1 ^a	0.1 ± 0.1 ^a	0.6 ± 0.5 ^a	1.0 ± 0.8 ^a
Cooked vegetables ^b	0.4 ± 0.4 ^a	1.8 ± 0.7 ^a	15.2 ± 3.5	45.4 ± 5.0	61.0 ± 4.8	60.7 ± 5.1	69.2 ± 4.2	62.9 ± 5.7	62.8 ± 4.4	62.7 ± 4.4	57.9 ± 4.1	55.8 ± 4.3
Raw vegetables	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.8 ± 1.9 ^a	6.1 ± 1.9 ^a	9.5 ± 2.3	8.6 ± 2.7 ^a	15.6 ± 4.5	21.7 ± 4.0	24.7 ± 4.6	16.5 ± 2.3	24.0 ± 3.5
Types of vegetables ^c												
Dark green vegetables ^d	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.6 ^a	10.9 ± 3.6	10.9 ± 3.2	6.5 ± 1.8 ^a	12.6 ± 4.5	8.7 ± 2.6 ^a	11.4 ± 3.1	16.6 ± 4.1	14.9 ± 3.9	7.9 ± 1.8
Deep yellow vegetables ^e	0.8 ± 0.7 ^a	20.5 ± 4.3	36.0 ± 4.9	30.3 ± 4.1	24.4 ± 4.5	15.1 ± 3.6	20.7 ± 5.1	15.4 ± 4.7	12.5 ± 2.5	15.9 ± 4.1	11.6 ± 2.8	16.0 ± 3.6
White potatoes	0.4 ± 0.4 ^a	0.6 ± 0.4 ^a	5.4 ± 2.5 ^a	20.1 ± 3.6	32.4 ± 4.6	29.6 ± 5.0	26.1 ± 4.2	31.5 ± 5.6	36.2 ± 4.4	33.4 ± 4.4	21.6 ± 3.1	31.1 ± 3.9
French fries and other fried potatoes	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6 ^a	6.3 ± 2.3 ^a	18.5 ± 3.8	11.6 ± 3.7	13.8 ± 3.2	16.7 ± 3.7	17.6 ± 3.5	20.6 ± 4.1	14.0 ± 2.8	21.4 ± 3.5

Table 1. Continued

	0–5 months		6–11 months		12–23 months		24–35 months		36–47 months	
	0–3.9	4–5.9	6–8.9	9–11.9	12–14.9	15–17.9	18–20.9	21–23.9	24–29.9	30–35.9
Other starchy vegetables ^f	0.1 ± 0.1 ^a	1.8 ± 0.9 ^a	12.4 ± 3.5	12.6 ± 2.7	11.8 ± 2.1	22.8 ± 4.3	17.9 ± 4.5	22.6 ± 4.6	16.3 ± 3.1	14.5 ± 2.2
Other vegetables ^g	0.3 ± 0.3 ^a	8.1 ± 3.3	23.9 ± 4.9	28.4 ± 4.4	26.8 ± 3.7	34.3 ± 4.4	36.2 ± 5.3	35.2 ± 5.6	32.7 ± 4.4	31.8 ± 4.0
Sample size	216	166	249	256	243	251	219	212	365	371

Data from 2008 FTTs.

^aPoint estimate is considered imprecise because of small sample size and uncommon or very common event.

^bIncludes 100% vegetable juice.

^cIncludes commercial baby food, cooked vegetables, and raw vegetables.

^dReported dark green vegetables include broccoli, spinach and other greens, and romaine lettuce.

^eReported deep yellow vegetables include carrots, pumpkin, sweet potatoes, and winter squash.

^fReported starchy vegetables include corn, green peas, immature lima beans, black-eyed peas (not dried), cassava, and rutabaga.

^gOther reported vegetables include artichokes, asparagus, beets, Brussels sprouts, cabbage, cauliflower, celery, cucumber, eggplant, green beans, lettuce, mushrooms, okra, onions, pea pods, peppers, tomatoes/tomato sauce, wax/yellow beans, and zucchini/su

Table 2. Percentage of children consuming meat or other protein sources at least once in a day ($\pm$ standard error)

	0-5 months			6-11 months			12-23 months			24-35 months			36-47 months	
	0-3.9	4-5.9	6-8.9	9-11.9	12-14.9	15-17.9	18-20.9	21-23.9	24-29.9	30-35.9	36-41.9	42-47.9		
Any meat or protein source	0.0 ± 0.0	3.1 ± 2.0 ^a	38.5 ± 5.4	76.9 ± 3.7	91.4 ± 2.3	91.0 ± 2.8	97.6 ± 1.0 ^a	96.1 ± 1.3 ^a	85.5 ± 3.3	97.4 ± 0.7 ^a	89.8 ± 2.2	95.2 ± 1.2 ^a		
Baby food meat	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 1.9 ^a	1.2 ± 0.5 ^a	2.1 ± 0.9 ^a	0.9 ± 0.6 ^a	0.7 ± 0.4 ^a	0.2 ± 0.2 ⁻	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
Non-baby food meat	0.0 ± 0.0	0.0 ± 0.0	3.6 ± 1.3 ^a	33.1 ± 5.0	60.4 ± 4.8	74.9 ± 3.7	76.2 ± 4.2	76.1 ± 4.7	65.3 ± 4.2	69.9 ± 4.2	69.4 ± 4.1	71.1 ± 3.6		
Other protein sources	0.0 ± 0.0	0.2 ± 0.2 ^a	12.6 ± 3.7	32.3 ± 4.5	59.4 ± 4.5	60.8 ± 5.3	65.2 ± 4.7	70.0 ± 5.2	57.5 ± 4.5	56.8 ± 4.6	62.0 ± 4.2	65.4 ± 3.8		
Dried beans and peas, vegetarian meat	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 1.6 ^a	2.0 ± 0.8 ^a	12.5 ± 3.5	11.5 ± 3.4	14.5 ± 5.3	11.4 ± 3.4	9.6 ± 3.4	9.3 ± 3.2	5.9 ± 1.8	15.2 ± 4.2		
substitutes														
Eggs	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.5 ^a	8.8 ± 2.9	14.7 ± 3.5	28.9 ± 4.6	22.1 ± 4.9	17.1 ± 3.2	14.1 ± 3.1	21.7 ± 4.2	18.8 ± 4.0	18.8 ± 3.5		
Peanut butter, nuts, and seeds	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1 ^a	3.5 ± 2.3 ^a	2.4 ± 0.9 ^a	3.2 ± 1.5 ^a	7.3 ± 4.0 ^a	4.6 ± 1.9 ^a	6.5 ± 1.8	5.2 ± 1.2 ^a	7.6 ± 1.7	10.4 ± 2.9		
Cheese	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 1.7 ^a	13.1 ± 3.7	21.0 ± 4.5	24.8 ± 4.1	31.0 ± 5.3	31.6 ± 5.0	26.0 ± 3.9	25.4 ± 3.8	30.6 ± 4.1	24.7 ± 3.5		
Yogurt	0.0 ± 0.0	0.2 ± 0.2 ^a	7.0 ± 3.1 ^a	11.2 ± 1.9	25.0 ± 4.3	17.8 ± 3.1	24.4 ± 4.3	27.8 ± 5.8	22.1 ± 3.1	20.8 ± 3.1	22.7 ± 3.7	21.6 ± 3.7		
Protein sources	0.0 ± 0.0	2.9 ± 2.0 ⁻	25.8 ± 5.0	42.4 ± 5.0	33.2 ± 4.6	24.5 ± 4.7	22.7 ± 4.4	24.6 ± 5.2	15.2 ± 2.5	34.5 ± 4.7	21.0 ± 4.0	17.7 ± 3.7		
in mixed dishes														
Sample size	216	166	249	256	243	251	219	212	365	371	395	330		

Data from 2008 FITS.

^a Point estimate is considered imprecise because of small sample size and uncommon or very common event.

^b Includes baby yogurt.

^c Includes baby food and toddler dinners as well as mixed dishes such as beans and rice, chili, pasta dishes and soup.

Table 3. Percentage of children consuming desserts, sweets, salty snacks and sweetened beverages atleast once in a day

	0-5 months			6-11 months			12-23 months			24-35 months			36-47 months	
	0-3.9	4-5.9		6-8.9	9-11.9		12-14.9	15- 17.9	18-20.9	21-23.9	24-29.9	30-35.9	36-41.9	42-47.9
Any Type of Dessert, Sweet, or														
Sweetened	0.3 ± 0.2 ^a	4.8 ± 2.2 ^a		17.0 ± 4.5	43.0 ± 5.0		62.8 ± 4.6	68.1 ± 4.9	74.0 ± 4.7	80.6 ± 4.8	86.0 ± 3.3	78.6 ± 4.8	87.2 ± 2.9	91.4 ± 2.7
Beverage														
Desserts and	0.0 ± 0.0	4.2 ± 2.1 ^a		11.2 ± 2.1	35.9 ± 4.8		53.9 ± 4.8	51.4 ± 5.1	59.2 ± 5.4	67.6 ± 5.3	70.3 ± 4.5	65.5 ± 4.7	72.0 ± 4.0	76.4 ± 3.8
candy														
Baby food	0.0 ± 0.0	1.2 ± 1.2 ^a		2.8 ± 1.0 ^a	11.9 ± 3.9		2.0 ± 1.0 ^a	0.3 ± 0.3 ^a	0.9 ± 0.9 ^a	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
desserts														
Cakes, pies,	0.0 ± 0.0	0.7 ± 0.7 ^a		7.9 ± 1.8 ^a	22.5 ± 3.6		40.4 ± 4.9	42.0 ± 4.9	36.6 ± 4.7	39.3 ± 5.6	50.4 ± 4.6	46.9 ± 4.5	46.2 ± 3.9	54.8 ± 4.3
cookies, and														
pastries														
Ice cream,	0.0 ± 0.0	1.4 ± 1.4 ^a		0.9 ± 0.7 ^a	6.5 ± 3.2 ^a		10.3 ± 3.4	9.7 ± 2.9	13.3 ± 4.0	14.6 ± 4.6	13.0 ± 2.7	9.9 ± 2.0	15.3 ± 2.7	18.2 ± 3.3
frozen yogurt,														
pudding														
Other	0.0 ± 0.0	0.0 ± 0.0		0.4 ± 0.3 ^a	2.4 ± 1.2 ^a		3.0 ± 1.2 ^a	5.2 ± 2.5 ^a	7.8 ± 3.4 ^a	5.2 ± 2.4 ^a	7.2 ± 1.9	10.2 ± 3.2	10.3 ± 3.3	9.0 ± 2.0
desserts														
Candy	0.0 ± 0.0	0.8 ± 0.8 ^a		0.0 ± 0.0	0.5 ± 0.4 ^a		6.6 ± 2.4 ^a	6.3 ± 1.7 ^a	20.1 ± 4.7	31.8 ± 5.5	25.2 ± 3.8	21.4 ± 2.8	29.4 ± 3.7	28.4 ± 3.8
Other Sweets	0.3 ± 0.2 ^a	0.0 ± 0.0		1.2 ± 0.9 ^a	6.5 ± 2.5 ^a		7.7 ± 1.8 ^a	15.8 ± 3.8	17.1 ± 4.4	21.9 ± 5.0	22.6 ± 3.4	20.4 ± 2.8	23.3 ± 3.7	28.7 ± 3.8
Milk	0.0 ± 0.0	0.0 ± 0.0		0.0 ± 0.0	1.4 ± 1.4 ^a		0.9 ± 0.7 ^a	0.9 ± 0.6 ^a	3.0 ± 1.5 ^a	3.7 ± 1.6 ^a	4.2 ± 1.3 ^a	3.3 ± 1.3 ^a	3.0 ± 0.8 ^a	7.3 ± 2.6
flavorings														
Sugar, syrup,	0.3 ± 0.2 ^a	0.0 ± 0.0		1.2 ± 0.9 ^a	5.2 ± 2.1 ^a		6.8 ± 1.6 ^a	15.0 ± 3.8	14.4 ± 4.2	18.3 ± 4.8	19.1 ± 3.2	17.8 ± 2.5	21.0 ± 3.7	24.1 ± 3.3
preserves														
Sweetened	0.0 ± 0.0	0.6 ± 0.6 ^a		5.0 ± 4.3 ^a	10.7 ± 3.2		14.3 ± 3.0	29.4 ± 5.1	28.6 ± 5.5	38.2 ± 5.6	41.0 ± 4.6	46.7 ± 4.5	38.5 ± 4.3	57.7 ± 4.1
Beverages														
Carbonated	0.0 ± 0.0	0.0 ± 0.0 ^a		0.0 ± 0.0	2.1 ± 1.5 ^a		1.4 ± 0.8 ^a	1.6 ± 0.8 ^a	4.2 ± 1.6 ^a	10.8 ± 4.7	8.1 ± 3.1	6.3 ± 1.5	5.3 ± 1.3 ^a	12.9 ± 2.5
sodas														

Fruit-flavored drinks	0.0 ± 0.0	0.6 ± 0.6 ^a	5.0 ± 4.3 ^a	6.3 ± 2.6 ^a	12.0 ± 2.9	22.8 ± 4.9	22.5 ± 5.4	23.0 ± 3.8	29.3 ± 4.5	37.0 ± 4.3	32.2 ± 4.3	39.8 ± 4.3
Sport drinks	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 1.2 ^a	0.7 ± 0.7 ^a	3.9 ± 1.6 ^a	0.3 ± 0.2 ^a	1.6 ± 0.9 ^a	3.0 ± 2.5 ^a	2.2 ± 0.9 ^a	0.7 ± 0.6 ^a	1.3 ± 0.7 ^a
Sweetened tea and coffee	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1 ^a	1.3 ± 0.7 ^a	3.3 ± 2.2 ^a	2.2 ± 1.3 ^a	4.1 ± 1.7 ^a	9.5 ± 3.5	4.6 ± 1.6 ^a	4.8 ± 1.4 ^a	10.2 ± 3.5
Other	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 1.3 ^a	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 1.7 ^a	2.8 ± 1.6 ^a	1.4 ± 0.7 ^a	1.0 ± 0.4 ^a	0.9 ± 0.4 ^a	1.6 ± 0.7 ^a
Salty snacks ^b	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1 ^a	2.3 ± 1.1 ^a	10.4 ± 2.7	16.5 ± 3.9	14.8 ± 4.8	23.7 ± 5.2	24.8 ± 4.1	22.9 ± 3.8	30.9 ± 3.8	29.0 ± 3.6
Whole grain	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1 ^a	1.4 ± 0.9 ^a	2.4 ± 1.4 ^a	0.6 ± 0.4 ^a	3.4 ± 1.6 ^a	6.7 ± 2.8	10.2 ± 3.3	7.5 ± 2.1	5.3 ± 1.6 ^a
Sample size	216	166	249	256	243	251	219	212	365	371	395	330

Data from 2008 FITS.

^aPoint estimate is considered imprecise because of small sample size and uncommon or very common event.

^bIncludes potato chips, popcorn, cheese curls/puffs, tortilla chips, and other types of chips and salty snacks.

usually in the form of yogurt or baby food dinners. Similar to the growth pattern observed regarding fruit in the infant diet, meats and proteins surged in importance at the end of the first year (over 75%), by which time the majority of children had graduated to eating non-baby food meats. From 12 months until the end of the 4th year, 90% of children eat at least some protein on any given day. From the age of 9 months, the leading meats were chicken and turkey, which together represented the meat intake of $21.7 \pm 4.4\%$ of 9- to 11.9-month-old infants; for 2- to 4-year olds, chicken and turkey were consumed by 36–47% of children. Hot dogs, sausages and cold cuts were consumed predominantly by toddlers and children at slightly lower levels than chicken and turkey (data not shown). In addition to discrete types of meats, 20% of toddlers between 12 and 47.9 months of age consumed meat in mixed dishes such as soups or vegetable/rice/meat mixtures. Approximately two thirds of toddlers and children through age 4 consumed non-meat sources of protein daily, chief among them yogurt and cheese (but also including meat substitutes, eggs and nuts), which contributed considerably to the protein levels among children in their 2nd year.

Sweets, Sweetened Beverages, and Salty Snacks

Shown in table 3 is the rapid increase in consumption of sweets and sweetened beverages from infancy to the 4th year of life. From 0 to 5.9 months of age, there is almost no considerable intake of sweets among infants, with a small proportion of children eating either baby food desserts or ice cream. By 6–9 months of age, nearly one fifth of infants consumed a sweet on any given day, primarily in the form of cookies and fruit-flavored drinks. Within 3 months, this value nearly triples to 43%, and within another year it doubles again to 80% at the end of the 2nd year, only to climb further into the 90% range by age 4. In 2008, one third of toddlers and young children were reported to consume candy. Although there was a 2-fold increase in the intake of salty snacks from the beginning to end of the 2nd year, these snacks contributed no considerable percentage of whole grain consumption to the children's diet. Lastly, sweetened beverages (including carbonated sodas and fruit-flavored drinks) were consumed by 10% of older infants, 38% of older toddlers, and 58% of 3-year-olds; approximately 10% of children over one year of age consumed soda at least once per day.

Discussion

Developing good eating habits early in life is important for one's long-term health status. [7–9] The FITS 2008 data illustrate that some positive changes have occurred since the last survey was conducted in 2002 while concern still exists in other areas. Of great importance is what appears to be a longer duration of breastfeeding followed by a delay in the introduction of complementary

foods. The 2002 FITS revealed that breastfeeding occurred in just 40, 26 and 21% of infants aged 4–5.9, 6–8.9 and 9–11.9 months, respectively [3]. In 2008, however, levels of breast milk consumption in these same age groups were 42, 33 and 33%. This pattern of a longer duration of breastfeeding has also been documented by others for the same time period [10]. Both a longer duration of breastfeeding and a delayed introduction of complementary foods are positive trends given their suggested role in the development of childhood obesity [11, 12]. The American Academy of Pediatrics (AAP) recommends exclusive breastfeeding throughout the first 6 months of life but also states that the introduction of complementary foods can occur around 4–6 months when the infant is developmentally ready [13, 14]. The 2008 survey shows that more children are beginning complementary foods at 6 months than in 2002. For example, data from the 2002 FITS showed that 29% of infants younger than 4 months were introduced to complementary foods, and this number was negligible in 2008. Of those aged 4–5.9 months in 2002, 65% consumed any infant cereal, 39% consumed fruit and 36% consumed a vegetable [3]. In 2008, each of these levels was lower (52, 22 and 26%, respectively); these percentages therefore suggest a more appropriate pattern of complementary food introduction in 2008.

On the other hand, not consistent with AAP guidelines was the continued use of cow's milk prior to 1 year of age, with 17% of children falling into this category in 2008, as compared with 20% in 2002 [2]. In 2008, approximately 20–30% of children were fed reduced fat milk on a daily basis during their 2nd year, which also conflicts with the recommendations of the AAP [13]; in 2002, 20–40% of children consumed reduced fat milk as toddlers [2]. While AAP recommends whole milk for children 12–24 months old [13], the American Heart Association recommends 2% milk for children in this age group [15]. This conflicting recommendation may make it hard for parents to know which type of milk to feed their child. It is important to keep in mind, that whatever the source of milk, ensuring adequate intake of both total fat and essential fatty acids in the diets of toddlers requires special effort and attention.

Foods rich in iron are recommended to be introduced around 4–6 months of age. [16] Infant cereal, a food that meets this need, was seen to be consumed in 2008 by a lower percentage of infants in two age groups, 4- to 5.9 (50.4%)- and 9- to 11.9 (51%)-month-olds, compared to the 2002 survey; 64.8% of 4- to 5.9-month olds and 63.8% of 9- to 11.9-month-olds [2]. While delaying the introduction of any complementary food is appropriate for the younger age group, a lower consumption in the older age group is of concern given the inadequate intake of iron in this age group reported by Butte et al. [17], which implies that other rich sources of iron are not being consumed by infants in this age group. This was indeed the case since a significantly lower percentage of infants in this age group were reported to be consuming baby meats. Furthermore, 43% of infants in this age group were consuming non-infant cereals in 2008, consistent with a level of 44% from 2002 [2]. While

non-infant cereals can be used to encourage the development of certain feeding skills, they contain less iron on a per gram basis as infant cereal [18].

The interpretation of how well parents adhere to recommendations regarding fruits and vegetables is more difficult to interpret. While we see a significantly lower percentage reporting consumption of any 100% juice as well as any fruits and vegetables at 4–5.9 months of age – another characteristic of the 2008 data which is in line with delaying the introduction of complementary foods – and a lower percentage of infants 6–11 months of age consuming any 100% juice with a sustained percentage of any fruit consumption, there was still a substantial proportion of infants and toddlers who did not consume any fruit or vegetable in a given day. The proportion not consuming any vegetables was even greater than that of fruits which is consistent with studies showing a preference of fruits at this age [19, 20]. In both 2002 and 2008, about 30% of older infants did not consume a vegetable each day, while about 25% did not eat fruit [2]. This finding of less than adequate fruit and vegetable intake among infants and toddlers is in line with others [21, 22] and is of concern given the World Health Organization's conclusion that dietary habits from childhood through adulthood could impact one's risk of cancer [23].

On a positive note, significant reductions in the percentage of infants and toddlers consuming any desserts or candy were seen in 2008. This pattern existed among children aged 6–20.9 months but disappeared starting around 21–23 months. In 2002, for instance, 46% of infants 7- to 8-months-old consumed a sweet, whereas in 2008 only 17% of those 6- to 9-months-old ate a sweet at least once a day [1]. In a similar manner, reductions in the percentage consuming sweetened beverages were observed (data from 2008 reflect a consistent decrease of 10% among 12- to 20.9-month-old toddlers), and for salty snacks among 4- to 11.9-month-olds [2]. Reductions in these foods which are contributors of discretionary calories are appropriate for these age groups.

Conclusions

The newest data from FITS appear to indicate that parents/caregivers may have taken to heart the advice of healthcare providers and public health messages that resulted from the publication of the 2002 survey. These positive changes include the longer duration of breastfeeding, a delay in the introduction of complementary foods, and a lower percentage of infants and toddlers consuming fruit juices, desserts, sweets, sweetened beverages, and salty snacks. There are, however, still concerns regarding low iron intake for 9- to 11.9-month-old infants, low fruit and vegetable intake for all children from 6 months to 4 years of age, as well as concerns over the use of cow's milk prior to one year of age and the use of reduced fat milks in the 2nd year of life. Furthermore, it appears that while we have made strides in the first year of

life, and to some extent in the 2nd year of life, the dietary habits of children after 12 months, and especially 2–4 years of age, are still reflective of the diets of older children and adults. [24–26] We now need targeted messaging to parents to keep monitoring and making wise food choices for their children as they grow older given that adequate and high-quality foods during the early years are of paramount importance for one's overall health and for the development of healthy eating habits [8, 23].

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Discussion

Dr. Stettler: I have one clarification and one question. The clarification is about fruits and vegetables in infants and toddlers. Was the percentage that you showed for children who consumed full servings as defined by USDA?

Dr. Siega-Riz: No it's any, it's not full servings.

Dr. Stettler: Isn't the recommendation before age 2 years of less than 5 servings of fruits and vegetables?

Dr. Siega-Riz: It's 5.

Dr. Stettler: Do you have any data on flavored milk?

Dr. Siega-Riz: We do have data on flavored milk, and actually it is in the article in the symposium. But I don't have those, there are too much data to show, but flavored milk is in there.

Dr. Villalpando: In the early stages, can you tell if it's full breastfeeding or any breastfeeding in the first 6 months of age?

Dr. Siega-Riz: We do have the data on exclusive breastfeeding. That is right now actually being analyzed so I don't have those data because you have to take into account the other. That's based on self-reports, so we can't verify that it is exclusive breastfeeding. The data that I am showing to you are actually on any breastfeeding, and it could have been mixed feedings.

Dr. Pandey: This study was conducted in America. Does it mean that it was weighted for the American population?

Dr. Siega-Riz: Basically, this is a random sample of mothers who have given birth and children under the age of 5. What we end up doing then is based on those characteristics we use the Census data to actually assign weights to the different children in order that they are reflected back to the representative sample for the United States. We do that for 2002 as well as 2008 so that it reflects what is happening at those two different time points. So, you do get the fact that we have two different populations between 2002 and 2008, but they are reflective of what is going on in the United States because we are using Census data to actually weight the samples up.

Dr. Lack: One big question is have you or are you able to look at introduction of allergenic foods such as eggs, peanuts and so on in addition to milk? That information would be very valuable.

Dr. Siega-Riz: We have the information there, and this is the first pass at the analysis. Because we collected 24-hour recall data, we actually have the type of food, so we will be able to look at that.

Dr. Lack: That would be fascinating. The second question I have is, do you intend to go back to the same cross-sectional cohorts 2 or 3 years later to see whether their diet early on is predictive of diet a few years down the line? These patterns might be set very early.

Dr. Siega-Riz: You have to understand that these are cross-sectional. I don't believe that they actually collected information that would allow them to go back. That was not necessarily one of their intentions.

Dr. Agarwal: Did you find out whether in the families where food and green vegetables consumption was low parents were purchasing green vegetables and fruits? Were the parents consuming green vegetables and fruits themselves?

Dr. Siega-Riz: We did not actually ask parents about parent's about their consumption, so we can't necessarily compare the parents versus the children.

Dr. Ganguly: I have two small questions. First, did you stratify the diet patterns according to the socioeconomic class, and were there any noticeable differences? Second, are there any cultural differences in the consumption of cow's milk in children less than one year of age?

Dr. Siega-Riz: We looked at women who participate in the WIC (Women, Infants and Children) program which is a program in the United States that provides food assistance to individuals below 185% of poverty, so it's considered a low-income group. We have done the analysis to look at differences between the participants of WIC in 2002 versus those in 2008, and we see similar changes. In fact, we are seeing the same sort of positive outcomes in our lower income population. The second question was whether or not we looked at differences in cow's milk by culture. We haven't necessarily done that. Unfortunately, I would say that once you start stratifying the sample by different ethnicities, there are going to be such small samples that you are not going to be able to tell very much. But we are interested in looking at maybe non-White, maybe Hispanic, to be able to understand some of those differences, but even there we are going to have to group the ages to be able to look at difference across ethnic groups. Dr. Reidy, did you want to mention something?

Dr. Reidy: Yes, as Dr. Siega-Riz said, looking at the WIC and the non-WIC populations, the changes over time have been similar in those two populations. But there are some very interesting differences with WIC children who are the lower income children in the US: significantly less breastfeeding, significantly less infant cereal, we saw a drop off in infant cereal after 8 months, that drop off is even bigger in the WIC children. We saw more whole milk being introduced before a year, even less vegetable consumption than in the general population and even more white potatoes, so there are several trends that are pretty concerning that are different in that WIC population.

Dr. Klish: I know that 50% of your subjects were college educated and the other 50% were not. Were you able to analyze who is getting the messages based on level of education?

Dr. Siega-Riz: We haven't done those analyses as of yet, by maternal education status.

Dr. Kleinman: And just to clarify, this survey was done probably just before the changes in the WIC food package.

Dr. Siega-Riz: They were definitely done before the changes in the WIC food package. The other thing to remember is the fact that WIC has a low prevalence of breastfeeding to begin with anyway.

Dr. Mohanty: I would like to know what the ethnicity of these children was, were they American Whites, African-Americans, American Indians? And question two is: has there been any change in feeding habits over these 8 years?

Dr. Siega-Riz: I am not so sure that there have been changes in feeding habits. The characteristic of the population in 2008 were as follows: 56% are non-Hispanic white, 14% are non-Hispanic black, 21% are Hispanic, and you also see that 30% of the population belongs to WIC, 35% are first born, and 46% have a college education or higher. As I said, because we weight the sample back to the United States Census, in fact there are differences between 2002 and 2008, and so we know that there are differences in our population at those two time points. The purpose of this paper was to basically show that there are different trends, and so in fact we are showing different trends and the kids are getting fed between the two time periods.

Dr. Mohanty: What is it that changes their feeding pattern? Is it recommendations from the American Academy of Pediatrics or some other agency in America?

Dr. Siega-Riz: I am not a pediatrician, I am a nutrition epidemiologist and I am a registered dietician. What I can tell you is the American Academy of Pediatrics sort of makes the consensus that really trickles down. There is a lot of different agencies that sort of adopt that recommendation, that actually help promote it. So, I think you are actually seeing it being promoted in many different sort of avenues, and parents have heard from the pediatricians, parents have heard from the nutritionists, it has been in the lay public, the institute of medicine has had different kinds of reports on obesity among our children and has actually brought some of these issues up to the public.

Dr. Mohanty: Are media advertisements influencing a lot?

Dr. Siega-Riz: Dr. Stettler, do you have anything to say about media advertisements?

Dr. Stettler: For those who are not based in the US, there are very good dietary guidelines for Americans but all of them, so far, have been age 2 and above. So below age 2 what people usually use are the recommendations of the American Academy of Pediatrics, so to answer your question, that's the authority in the US for nutrition up to age 2. After age 2, the US Department of Agriculture has dietary guidelines. Now regarding exposure to media, there is very little regulation in the US. There has been a change recently within industry self-regulation of advertising to children but that may have been after 2008, I don't remember, it's around that same period (<http://www.bbb.org/us/children-food-beverage-advertising-initiative/>).

Dr. Siega-Riz: I must admit, because of the emphasis on childhood obesity in our country, there are many programs, and there are many community programs that have trickled down to head start programs for lower income children that are actually sort of adopting these recommendations in order to get kids to eat healthier. So, I would imagine that if we are to do this again, I would hope that we would actually see some more of these positive trends.

Dr. Kleinman: There have been a number of surveys to look at what parents listen to when it comes to medical advice and dietary guidelines. Physicians remain highly regarded sources of this information, but have less influence because of the many other sources available to parents, through the internet and media. Advertising is highly influential and of course we know that children spend an inordinate amount of screen time each day.

Dr. Siega-Riz: And the one thing I want to add, unfortunately I don't have the slides from the talk that we gave at the American Academy of Pediatrics, but in this survey we were able to ask the question of how many of these children actually have a TV in their room and how many hours of television and screen time they were doing. In fact, I dare to say, even under the age of 2 it's an incredible number of how many kids

are being exposed to television and they are actually being fed in front of television. So, I think it's becoming more prevalent and clearly an area of concern.

Dr. Birch: Can you provide information about differences between the 2002 and the 2008 FITS samples?

Dr. Siega-Riz: There are differences in who participated, but once we weighted up to the United States population, we were actually weighting it up and we were actually describing what's happening in America at those two different time points. So, what's happening then between 2002 and 2008 is not necessarily due to the differences in the population but it's describing really what's happening, how they are being fed. And we are seeing that in 2008 we have more Hispanics, we have actually some more higher educated individuals, but that's what's happening in our population.

Dr. Lack: I understand that you are saying the change in demographics or characteristics you don't believe explain some of the changes in diet that you have seen, but I just wonder how generalizable your data are to the population because I see 46% of mothers have a college degree or higher, that sounds like a very high level of education. You could argue that the changes in the 6 years took place because you are looking at a very educated population that will take on public health messages more.

Dr. Siega-Riz: Let me take this a little bit further. We have 46% of the mothers in the sample that got surveyed, but then when we weighted it up to the United States, you actually end up downweighting that number because there is less higher educated people. When you create the weights, you are actually reflecting it back to who is living in the United States, so even though you have greater higher educated women in your sample, you are downweighting them to make them representative to who is living in the United States at that time point. That's the difference.

Dr. Lack: I understand, that answers my question. Related to that, it would be interesting to see if you compare 2002 and 2008 in the college-educated versus non-college-educated families whether that might give you an idea of the cause of this being education, whether you see a difference in one group and not in the other.

Dr. Siega-Riz: We haven't at all touched the data yet to understand what the determinants of these changes are; and that's what we need to do, it's to be able to understand the changes in these behaviors and what's driving those changes, and we haven't done the analysis to answer that question yet.

Dr. Jones: Was the sampling done on a week-end or a week day, because there could be a difference in dietary intake? In many parts of the world, there is quite marked seasonal variation, particularly in fruit and vegetable intake, and so was the sampling frame over a whole 12 months or was it in a particular season?

Dr. Siega-Riz: In fact, the dietary intake was one day of dietary intake among all the children and then a small subgroup actually had a second day of dietary intake, and they were mixed between week days and week-ends. And as far as the season is concerned, I don't remember. Dr. Reidy, do you remember what the season was? I thought that the season was supposed to mimic what we had in 2002.

Dr. Reidy: Actually, the 2002 data were collected in spring-summer, and this was more summer-fall. So, they overlapped quite a bit, but they were a little bit different. Some of the literature that we had consulted indicated that there weren't huge changes in young children in terms of dietary intake.

Dr. Jones: Mine has to do with vitamin K levels in the blood which are predominantly going to come from green vegetables, and there clearly is marked seasonal variation in vitamin K levels in Australia.

Dr. Siega-Riz: One of the things that we can look at, and you'll see that we actually did look at the data baby food versus table foods, is to see if there are some seasonal changes. But that's one of the other layers of analysis that we haven't necessarily done yet.

Dr. De Beer: I am very interested in your study because we have now done a couple of food consumption surveys in the country. My first question concerns the 24-hour recall methodology which in itself has got certain limitations, and perhaps you want to comment on that. The second question that I have is having potatoes as part of your vegetable group, because by taking that out I think the whole thing changes quite significantly. And the last thing that I want to ask is you mentioned that one of the reasons you saw some changes was based on pediatric recommendations and it related to the breastfeeding duration that was now increased. You said it was the recommendations from the pediatricians. My question to you is, have you actually asked the mums where they get the information from and what causes mums to do what they are doing?

Dr. Siega-Riz: So, 24-hour recall data actually are very good. They are the best data that we have for assessing dietary intake, it's better than a food frequency questionnaire, some would say. In fact, it allows us to provide more absolute levels of intake. The other thing we have to remember is that we are not necessarily looking at individual intakes, we are looking at group means, so one 24-hour recall to look at the group mean here was considered to be scientifically valid, and we did work with the mums to try to get that information for children who are in day care as well. That's probably one of the biggest stumbling blocks in collecting dietary information for children of this young age group. There is a lot of them who are spending time in child care. The second question, can you just remind me what that was?

Dr. De Beer: It was related to the potatoes.

Dr. Siega-Riz: As a nutritionist I agree with you. I like to sometimes be able to look at the data with or without potatoes to see really how much contribution it is. So my colleague Barry Popkin and I wrote this classic paper on adolescent nutrition that looked at changes in adolescent dietary behaviors from 1965 through 1994. We actually took out or looked at what proportion of vegetable consumption came from potatoes, and it was one third, it was huge. So, yes, in fact we could argue that perhaps we should be taking that out and looking at it. However, I will also argue that potatoes, if not fried, are a good source of vitamin C, so it's not necessarily something that we should totally get rid of from the diet, it has a place in the diet. I just wish it wasn't as prominent. And then the last one was?

Dr. De Beer: The pediatric recommendation.

Dr. Siega-Riz: I think, as my colleague alluded to, when you talk to women, and I talk to a lot of pregnant women because I actually do a lot of research in pregnant women and follow them up through the first year of life of the infants, they will actually tell you that they are influenced not only by their doctor but they are influenced by their mother and other family members and friends. So, it really depends on the cultural upbringing of that particular woman as to who is going to be the most influential person in her making that child feeding decision. I think we need to understand a little bit better about the culture and the culture that that woman has been brought up in to be able to understand. I don't remember if we actually asked the question, did we?

Dr. Reidy: No, we didn't, but I wanted to just comment on the recommendation piece. In 2002, it was just about when the AAP came out with their juice recommendation, and that got amazing press for many years after that. I really do think that the recommendation and then the way it was played out in the lay press, pros and cons of juice and how bad juice is, I have no evidence but it seems like that must have been a driving factor behind at least that one change that we saw, I think the timing was perfect for that.

Dr. Mace: Have you tried to stratify your data with the BMI of the parents?

Dr. Siega-Riz: No, and we don't have BMI of the parents. It would all be based on self-reports, so you can only imagine.

Mr. Fryer: With the trends in the 2008 survey of decreasing iron-rich infant cereal and increasing whole milk consumption, has there been any negative impact shown on iron deficiency in the US recently?

Dr. Siega-Riz: That's what I was actually commenting, that in fact these data we can also look at and it's not from a clinical measure, it's from diet and actually looking at the recommended intakes based on the Food and Nutrition Board in the United States. So, we can actually see that there was a slightly higher proportion of children from 9 to 11 months old that have lower intakes of iron. They were below the estimate average requirement, so that's what we were making that comment on.

Dr. Lack: Between 2002 and 2008, there might have been changes in the number of fathers taking care of their infants as a proportion and actually reporting the diet of their infants. Have you looked at the percentages between the two time points and specifically whether fathers report dietary intake in a different way to mothers or feed their children in a different way to mothers?

Dr. Siega-Riz: We do have the information on who the caregiver is, but we have not looked to see whether it's the mother or the father. You bring up some interesting things that would be nice to be able to study, and I am not aware of any studies that I can report. I don't know if anybody has studied differences in feeding behaviors, whether it's a mother or a father actually being the primary caregiver.

Dr. Pandey: Just a comment. We have learned that nutrition at periconceptual age is decisive in the child's food preferences later on in life. So if a child makes a particular food choice, is he/she guided by his/her taste or is it the parents' taste that decides or maybe the genetic predisposition? I think it is not so simple.

Dr. Siega-Riz: I think you are asking a question that a lot of us are very interested in teasing out. I do study maternal nutrition during pregnancy, and I can tell you from our cohort of over 5,000 pregnant women that we have had in North Carolina, and I have colleagues who have actually done similar studies in Massachusetts and Michigan and in Seattle, that we actually see that pregnant women are consuming about 35–40% of the calories from fat, that in fact you see they are not meeting their recommendations of fruits and vegetables and specially not for fiber, and you are also seeing a high consumption of sweetened beverages. When we look at differences by whether it's a first-time mum or a second- or a third-time mum, you actually see the first-time mum is more likely to watch what she is eating, and then as subsequent pregnancies come along, it relaxes a little bit more. We need more of those studies that we are able to then follow and correlate infant feeding behaviors between the mum in pregnancy and the child growing up. Some of us are doing that work, and I think you will actually get to see it. There are some studies that just look at parents feeding behaviors and how it correlates with their child, what the child is consuming, and you do see a pretty high correlation between the two.

Dr. Shreffler: Are you collecting any basic self-reported information about health status of the kids just to see how that might influence dietary choices, eczema, allergies and other problems?

Dr. Reidy: We did ask about a few things. We did ask about food allergies specifically, and then if the children had eaten normally that day or if they were sick.

Dr. Siega-Riz: So, it wouldn't be as detailed as I have seen in some of our cohort studies, where we looked at wheezing and coughing and development of asthma, and whether asthma was diagnosed, like the cohort studies Matthew Gillman and myself have done.

Weaning Practices in Other Parts of the World: Case Study India

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Abstract

Infant feeding and weaning practices in India continue to demonstrate that a significant number of infants do not receive colostrum (62.8% according to the National Family Health Survey, NFHS-2), though breastfeeding is universal and continued for a longer period. In NFHS-3 (2005–2006), there is improving trend for breastfeeding within the first hour of birth (23.4%) and exclusive breastfeeding up to 5 months (46.3%); however, weaning for semisolids is delayed (55.8% only at 6–9 months of age). The infant weaning foods are inadequate in energy-protein and micronutrients. Further, weaning foods and feeding/cooking utensils are contaminated with bacteria, resulting in frequent episodes of diarrhea. Indeed, these are the factors responsible for initiation and continuation of early malnutrition which the country has failed to control as observed in the three NFHS. Over a span of 7 years, i.e. from NFHS-2 (1998–1999) to NFHS-3, there was only marginal reduction in undernutrition. Thus, uncontrolled fetal malnutrition, poor initiation of breastfeeding, inadequate and delayed weaning, and contaminated food and water demand urgency to develop affordable hygienic weaning foods, education to clean utensils, timely weaning and available potable chlorinated water to prevent and control malnutrition.

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Introduction

A landmark decision to protect, promote and support breastfeeding was taken in the 54th World Health Assembly in 2001 giving rise to a Global Public Health recommendation for exclusive breastfeeding during the first 6 months of life, complementary feeding with home-based safe and nutritious foods to start at 6 months of age and continued breastfeeding up to the age 2 years and beyond.

The correct norms for infant and young child feeding include the following:

- Initiation of breastfeeding immediately after birth – preferably within 30 min.
- Exclusive breastfeeding for the first 6 months – the infant receives only breast milk and nothing else, no other milk, food, drink or water.
- Appropriate and adequate complementary feeding from 6 months of age while continuing breastfeeding.
- Continued breastfeeding up to the age of 2 years or beyond.

Weaning is the process of gradually introducing foods other than breast milk in a child's feeding schedule. This process starts when any food besides mother's milk is introduced in the child's diet, and is completed only when the child has been entirely put off the breast. The introduction of supplementary foods not only ensures the fulfillment of nutritional requirements but also introduces the child gradually to the normal family eating patterns. Infants are at greatest risk of diarrhea when foods other than breast milk are given first. This is because during weaning infants are being exposed to food-borne germs for the first time and they lose the protection of breast milk which has anti-infective properties. High levels of contamination are often found in animal milks and traditional weaning foods, especially prepared cereal gruels (rice, pulse, and semolina). *Escherichia coli*, which causes at least 25% of all diarrheas, is commonly found in weaning foods. Feeding bottles and rubber teats, which are particularly difficult to clean, are often breeding grounds for germs. There is a need for infants older than 6 months to receive more than just breast milk in order to grow well, balanced against the risk of getting sufficient energy, protein, vitamins and minerals, and the meals being prepared hygienically. It is important for health personnel to work with local communities to identify and encourage safe weaning practices and to improve infants' nutrition to increase their resistance to infections and prevent diarrhea [1, 2].

Infant Feeding Practices and the Growth Pattern

Earlier studies in 1976–1977 showed that colostrum was discarded by 95% of mothers; 81% started the first feed (74% cow/goat milk in 1:1 dilution) in the first 2 h; breastfeeding was initiated on the 3rd day in 72%; 92% rural and 76% urban slum women continued to breastfeed up to 12 months; complimentary milk was started around 3–6 months, and semisolids were given in 30% by the 1st year and 65% by the 2nd year of age [3]. Indian Academy Pediatrics conducted a nationwide survey in 1982–1983 (5,235 households) to observe that 40% discarded colostrum except in western India (Rajasthan, Gujarat and Maharashtra); breast milk was the first feed in 22% in north eastern and north western India, in other states it varied between 11 and

15%; 56% working women and 41% housewives started semisolids at <6 months of age, while around 20% Indian children did not receive any semisolids by 12 months of age (table 1). 60–70% women in urban areas used detergent and hot water for cleaning the infant feeding utensils, while >80% of rural and tribal women used mud/ash with water [4]. The weaning food samples (cooked rice, legume, bread, vegetable and sago) used for children aged 6–24 months were collected from Varanasi rural and urban slum areas for bacteriological studies; the samples were positive for *Escherichia coli* (indicating fecal contamination; 58%), *Klebsiella pneumoniae* (15.3%), *Pseudomonas aeruginosa* (18.7%), *Streptococcus fecalis* (14%), *Proteus* (2%) and *Citrobacter* (0.2%) [5]. Similarly in Chandigarh, weaning foods from middle- and high-income families were contaminated with *E. coli* in 66.8 and 8.5%, respectively [6].

The data from three National Family Health Surveys (NFHS) are summarized in table 2. There has been improvement in frequencies for breastfeeding within the first hour of birth, exclusive breastfeeding >4–5 months, and introduction of semisolids by 6–9 months of age. The NFHS-3 (2005–2006) showed that 46% children aged <3 years are undernourished; of these, 2.8% are acutely severely malnourished and will need hospital care. Over the 7 years that separated the NFHS-2 (1998–1999) from NFHS-3, there was a marginal reduction in malnutrition in children <3 years old, while wasting increased from 20 to 23%, and anemia by 4.7% (suggesting endemic deficiency of macro- as well as micronutrients). In NFHS-3 (as well as in earlier surveys) the important findings remained: 18% underweight; 19% stunted, and 24% wasted during the first month, rising further at 1 month of age; at 5 years the figures were 51, 55 and 21%, respectively (fig. 1) [7, 8], clearly indicating failure of health and nutritional programs. Early life undernutrition is the continuity of maternal-fetal undernutrition resulting in low birthweight babies. Figure 2 shows that fetal weight gain is poor during 36–42 weeks of gestation in rural India [9]. The babies of undernourished mothers (36% in NFHS-3) with intrauterine growth retardation had poor neuromotor development and disorganization of sleep pattern, suggesting dysmaturity of brain [10, 11]. Intrauterine growth-retarded babies of undernourished mothers had lowest means for weight, crown-heel length, skull circumference, and showed delayed development during 9 months of growth [12, 13]. The fetal and early life malnutrition affects higher mental functions, and shows persistence of soft neurological signs in school years, irreversible mental dysfunction(s) with poor fine motor coordination [14, 15]. In addition, the accompanying maternal anemia (>65%) in India [16] induces irreversible brain neurotransmitter alterations [17]. These are serious health consequences. The undernourished/anemic mothers are poorly prepared for breastfeeding and healthy weaning practices.

Table 1. Infant feeding practices according to the 1986 Indian Academy of Pediatrics national study report (survey 1982–1983)

Zones	Discarded colostrum	Breastfed on 1st day	Received compli- mentary milk at 1–3 months of age	Received semi- solids at <6 months of age	Did not receive semisolids at >12 months of age
East (n = 1,043)					
West Bengal, Orissa, Assam, Manipur	U = 33 R = 63 T = 73	38	31	41	U = 16 R = 14 T = 1
Central (n = 1,861)					
Bihar, Uttar Pradesh, Madhya Pradesh	U = 38 R = 36 T = 47	36	35	31	U = 21 R = 30 T = 13
South (n = 350)					
Andhra Pradesh, Kerala, Karnataka, Pondicherry	U = 40 R = 42 T = 0	46	26	46	U = 12 R = 12 T = 0
West (n = 1,272)					
Gujarat, Maharashtra, Rajasthan	U = 42 R = 40 T = 3	69	21	24	U = 16 R = 33 T = 84
North (n = 709)					
Punjab, Haryana, Himachal Pradesh Delhi	U = 38 R = 32 T = 0	62	39	47	U = 12 R = 29 T = 0

Figures indicate percentages. U = Urban; R = rural/tribal; SS = semisolids.

Table 2. Child feeding practices and nutritional status (NFHS-1, -2 and -3)

Feeding practices	NFHS-3 (2005- 2006)	Urban	Rural	No edu- cation	Education <5 years	Education 5-9 years	Education ≥10 years	NFHS-2 (1998- 1999)	NFHS-1 (1992- 1993)
Breastfed within 1 h of birth	23.4	28.9	21.5	15.9	27.7	29.1	33.0	16.0	9.5
Exclusively breastfed (0-5 months)	46.3	40.3	48.3	48.1	55.9	44.3	40.8	NA (55% <4 months)	NA
Children 6-9 months receiving solid or semisolid food + breast milk	55.8	62.1	53.8	49.1	51.5	58.4	69.6	35	NA
Children <3 years stunted	44.9	37.4	47.2	53.2	48.4	41.4	26.3	51.0	NA
Children <3 years wasted/ underweight	22.9/ 40.4	19.0/ 30.1	24.1/ 43.7	26.8/ 50.2	25.0/ 45.8	20.4/ 34.9	15.1/ 20.5	19.7/ 42.7	NA/ 51.5

Figures indicate percentages. NA = Data not available.

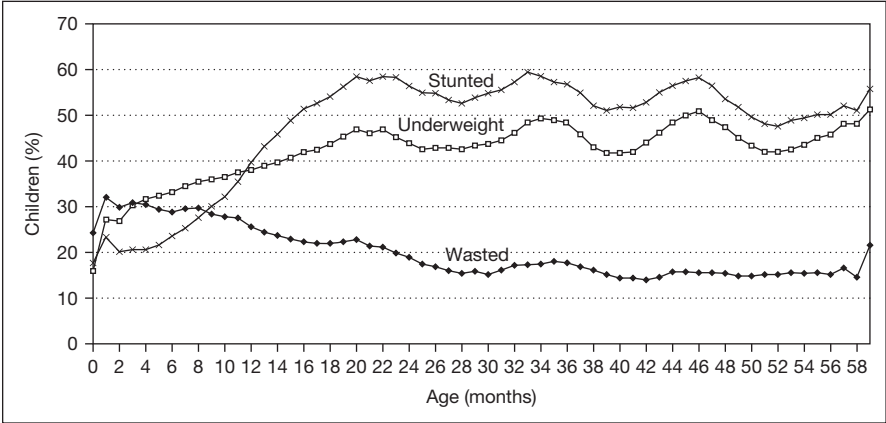


Fig. 1. Stunted, underweight and wasted children <5 years of age. NFHS-3 (2005–2006) data.

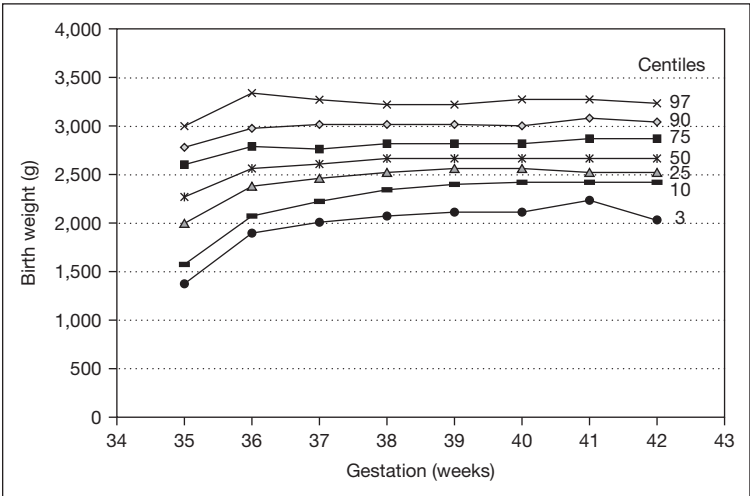


Fig. 2. Birthweight percentiles for gestation (rural Varanasi, n = 3,700). Among live births, 7.2% were <2,250 g and 27.4% <2,500 g. The weekly birthweight increments in gestation 36–42 weeks were 5–53 g only. From *Indian Pediatrics*, with permission.

Age at Weaning and Linear Growth

In the NFHS-2, length/height growth data for 2- to 4-year-old children were correlated with their age at weaning. It was found that those weaned at or after 6 months of age were more likely to be stunted at later age compared

with those weaned before 6 months ($p < 0.001$). Stunting appeared to be considerably lower for children weaned at age 3 months and showed an upward trend thereafter [18]. In contrast, studies on poor urban Chilean infants and term low birthweight infants (1,500–2,500 g) in Honduras showed that exclusive breastfeeding for 6 months is sufficient to support growth [19, 20]. Similarly, early introduction of complementary feeds in Vietnamese infants was associated with poorer growth [21].

Impact of Integrated Child Development Services

In 9- to 36-month-old children in an Integrated Child Development Services (ICDS) block of Delhi (in service for >20 years), situated 2 km away from the University College of Medical Sciences Hospital, dietary intake was 56% for energy (against RDA for age) only. As for their nutritional status, 75% were underweight (-2 SD), 35% were severely undernourished (-3 SD), 74% were short statured (severe malnutrition), 39% were severely stunted, 19% were wasted, and 10.0 and 9.8% had the peak of severe acute protein energy malnutrition (SAMN) at the ages of 31–36 and 13–18 months, respectively, with girls being more affected [22]. This situation occurred despite the nationwide coverage of ICDS providing food supplementation to pregnant and lactating women and children in Anganwari centers for over 2–3 decades [7, 8].

Suitable Supportive Weaning Food(s) for Infant Diet to Prevent and Control Malnutrition

India, mainly a vegetarian society where late and inadequate weaning diet subsisting on cereal and/or pulse diet, bacterial food contamination and unsafe potable water are common, is burdened with high prevalence and severity of malnutrition. There remains a need to develop protein sources with richness of minerals and vitamins to prevent/control protein-energy malnutrition with associated micronutrient deficiencies. Protein-energy malnutrition is the most frequent cause of secondary immune deficiency in children with significant impairment of cell-mediated and humoral immune responses.

The protein contents of common Indian leafy vegetables per 100 g eatable portion are: amaranth 2.5 g, mustard 2.7 g, turnip 1.5 g, broccoli 1.8 g, cauliflower leaves 2 g, and dried drum stick leaves (*Moringa oleifera*) 29 g. The fresh leaves of an Egyptian clover called berseem (*Trifolium alexandrinum*) contains 18–23% protein. However, the micronutrient content is similar in all leaves. Thus, berseem was developed as leaf protein concentrate (LPC) by ultrafiltration and acid thermocoagulation. 100 g of berseem contains 344 cal, 60 g protein, 22.5 g fat, 12.5 g CHO, 1 g fiber, 86,700 µg β -carotene, 0.5 mg vitamin B₁, 0.5 mg vitamin B₂, 24.2 mg vitamin B₅, 1 mg vitamin B₆, 330 mg

vitamin B₉, 4.3 mg pantothenic acid, 2.2 mg vitamin C, 1 mg vitamin K, 187 mg Ca, 604 mg P, 99 mg Fe, 9 mg Zn, 384 mg Mg, 2.1 mg Cu and 713 mg K.

Fermented milk/curd (dahi) is commonly taken in Indian diet, and 1 g contains 10⁸ CFU of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; 100 g contains 60 cal energy, 3.1 g protein, 4 g fat, 3 g CHO, 102 µg β-carotene, 0.05 mg B₁, 0.16 mg B₂, 0.1 mg B₅, 12.5 µg B₉, 1 mg vitamin C, 149 mg Ca, 93 mg P, 0.2 mg Fe, 130 mg K, 32 mg Na.

Children with SAMN were given LPC (n = 36) or dahi (n = 32); both supplements provided 6.0 g protein in each group, along with the WHO recommended diet for 15 days. There was an increase in serum proinflammatory (TNF-α, IFN-γ), and anti-inflammatory (IL-10) cytokines but a fall in IL-4 levels. The rise in IL-10 was significantly higher in the dahi diet group. There was an increase in the CD4:CD8 ratio after treatment in both groups [23, 24].

In view of the anti-inflammatory properties of fermented milk (dahi), SAMN children were given a milk diet recommended by the WHO, and in the other group dahi was fed in place of milk. These studies showed that the WHO milk diet further reduced the absolute lymphocyte count (ALC) and other components after 6 weeks of therapy. In contrast, the WHO dahi diet increased ALC, CD3+, CD4+, CD8+, CD19+, CD56+ lymphocyte counts. In addition, the level of IL-1, -6, and -10 increased significantly more with the dahi diet than with milk [unpubl.]. These studies demonstrate that dahi with its immune-nutrient properties should replace milk in the WHO milk diet to treat and control severe malnutrition. Dahi will also be an ideal weaning food in place of generally used cattle milk, as immune processes are developing at this age.

In the tribal belt of India, the introduction of appropriate amount of green leafy vegetable powder as a source of micronutrient in weaning food reduced malnutrition-linked mortality in infants [25].

In India, to prevent and control malnutrition, exclusive breastfeeding for 6 months is essential. The process of weaning must involve education about how to hygienically prepare weaning foods, clean infant feeding utensils and sterilize them in a pressure cooker. More and more women in towns and rural areas (construction workers) are in need of infant energy-, protein- and micronutrient-rich foods that are properly packed and at reasonable prices. The ICDS managers should urge food industry to prepare weaning foods based on recent researches on fermented milk (dahi), with proper hygiene and cold chain and cereal pulse mixtures with green leafy vegetables (LPC). Despite numerous maternal child health and nutrition programs, we fail in providing clean water. Good hygiene awareness should be inculcated and support for potable water by use of chlorine tablets promoted.

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Weaning Practices in Other Parts of the World: Case Study Russia

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Abstract

Objectives of the Survey: To evaluate infant feeding and weaning practices and anthropometric characteristics of 2- to 24-month-old children in Russia. **Survey Method:** A comprehensive analysis of data collected from face-to-face interviews of a random Russia representative sample of 2,500 mothers of children. We used a specially designed questionnaire that includes sections on health, especially feeding practices, food intake from the previous day and the measurement of height and weight. The survey was conducted within the framework of 'Start Healthy Stay Healthy' program in Russia, sponsored by Gerber, Nestlé Nutrition. **Results:** Incidence of breastfeeding among children of various age was as follows: from 2 to 4 months: 70%, from 4 to 6 months: 60%, from 6 to 9 months: 46%. The most common weaning food was fruit juice (59.4% of children), followed by fruit puree (18%) and cereals (6.4%). 4.4% of respondents used cow's milk as the first weaning food. It was found that examined children were slightly taller (z score for height-for-age, 0.11) and heavier (z score for weight-for-age, 0.63) than the WHO standards. **Conclusions:** Evaluation of infant/toddler feeding and weaning practice will help to develop guidelines and educational programs to prevent nutrition-related diseases in Russia.

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Infant feeding as a factor determining not only growth and development of the child, but its health in the later life, remains the center of nutritionist attention. Early ab lactation, wrong introduction of the supplemental feeding, using unadapted infant formulae and high level of protein consumption are the reasons contributing to the development of adiposity, hypertension, and glucose intolerance in the older age [1–3].

Studies previously conducted in Russia show various infant feeding practices. These studies were mainly devoted to certain aspects of infant nutrition,

and were conducted using limited (nonrepresentative) samples, and could not characterize the situation in the country as a whole [4–6]. That is why the objective of this work was epidemiologic evaluation of infant nutrition, incidence of breastfeeding, using adapted and unadapted infant formulae, introduction of weaning food, physical development evaluation, children's consumption of energy and nutrients.

The study was conducted by the Institute of Nutrition in cooperation with Institute of Sociology, Gerber and Nestlé within the program 'Start Healthy Stay Healthy' in Russia.

Material and Methods

A total of 2,582 children aged from 2 to 24 months (Russian representative sampling) were evaluated in 38 regions of Russia; 15.6% of children were aged 2–5 months, 30.0% of children were aged 6–11 months and 54.4% of children were aged 12–24 months. Boys comprised 51.6, girls 48.4% of the study sample.

For the purpose of the study, a questionnaire was formed, on the basis of which information about the family was obtained (mother's age and her education, composition and income of the family), and health, development and nutrition data. To evaluate the actual nutrition of the child, a 24-hour recall method was used; besides, parents of 30% children were questioned twice [7]. Anthropometric evaluation (body length and height) of the children to analyze their nutritional status was conducted using the WHO-ANTRO 2005 program.

To calculate chemical composition of the actual food rations, computer databases containing information about chemical composition of about 3,000 products and dishes, were used. The reference was based on chemical composition tables of domestic products [8], and for imported products, US, UK, and German chemical composition table data and labeling information were used.

While calculating chemical composition of nutritional rations, protein content in the breast milk was considered to be 1 g per 100 ml.

Statistical processing of the data was performed using SPSS 14.0 for Windows.

Results and Discussion

By the end of the first month, 82% children were breastfed, 18% of children received infant formulae and 2% of children were fed formula since birth. The incidence of breastfeeding, which was considered receiving not less than 200 ml of breast milk a day, was 70% from the age of 2 to 4 months, 60% from 4 to 6 months, 46% from 6 to 9 months, and 39% from 9 to 12 months; 16.9% of children older than 12 months also received breast milk. These data are in agreement with the results of other studies [4, 5, 6].

Breastfeeding of the child (for any time period) did not depend on mother's education, family income level, and place of residence.

A total of 1,659 children were fed formula, 18% from the first month, 55% from the 3rd, and 70% from the 4th.

The first weaning food was fruit juice (in 59.4% children), then fruit puree (18%) and porridges (6.4%). It should be noted that 4.4% of respondents used cow's milk as a first supplemental nutrition, and 1.1% kefir (fermented cows' milk).

As the second most common weaning food, 47.4% of children received fruit puree, then vegetable puree, juice, and porridges (14.9, 13.5 and 12.2%, respectively). Cow's milk and kefir as the second supplemental nutrition were received by 1.8 and 1.2% of children, respectively.

The results of the study showed that 2.5% children received cow's or goat milk from the first month of life, and 26% of the children from 4 months. At the 7th month, more than 50% were receiving milk. Kefir was started from 2 months in 2.3%, from 5 months in 23% of children, and from 7 months in 41% of children. Other fermented milk products (fermented baked milk, yogurt and others) were used: at 6 months by 11.5% of children and at 8 months by 40% of children; by the age of one year, these products appeared in the diet of more than 80% of children.

Analysis of weaning food showed that among small grain infant food, buckwheat, rice and oat porridges are preferred, while among products prepared at home, semolina is used more often. Among juices and fruit purees, both specialized and home cooked, apple juice is used most often – in 81%, and apple puree in 84.5%. Among manufactured vegetable purees, the most popular (34.4%) is vegetable mix; at home, potato is preferred –44%.

As a first manufactured weaning meat – 56% are fed chicken and 29% rabbit meat.

Among home-cooked meat products, 45% of children receive beef and 43% pork.

An association was found between mother's education and contribution of different products to the daily caloric value of the child's nutrition. For example, children whose mothers had higher or unfinished higher education, at the age of 2–5 months received authentically more (1.5-fold) energy with breast milk, and children aged 6–11 months received 1.5- to 6.8-fold more energy from specialized infant food (porridges, vegetable, fruit and meat canned goods; $p < 0.005$), compared to the children whose mothers did not have higher education. In contrast, children whose mothers did not have higher education received 1.4- to 3.2-fold more ($p < 0.05$) energy with milk and kefir, porridges, potato dishes, bread, and pastry compared to the children whose mothers had higher education.

The finding of early introduction of cow's milk and other unadapted milk products to the infant's diet is in agreement with other studies [4, 6, 9–11]. Taking into account the literature [1–3, 10, 12] and our own data [5] on possible negative effects of these products on infant health, it can be suggested that using these products may constitute a risk factor for iron deficiency and obesity. The revealed variations may be due to limitations of the system of

provision of free specialized infant products, and inadequate education of the population by pediatric specialists regarding infant nutrition.

This study revealed that the medical personnel did not make enough effort to educate parents about children nutrition. Parents said that about 20% of doctors and 40% nurses do not inform them about infant healthy nutrition rules. At the same time, relatives' advice and specialized literature were relevant. For 20% of parents, commercials were a source of information on infant nutrition. These data comply with the results of studies conducted earlier [4].

Analysis of anthropometric data showed that 64.4% of boys and 61.6% of girls had normal weight for their age and 51.3% of boys and 47.0% of girls had normal height for their age within one standard deviation from the standard population median (WHO ANTRO, v.2.0.4).

Z score exponents of growth by age showed that the proportion of nanous children (z score < -2) at the age of 2 months to 2 years was 4.0–14.5% for boys, and 5.0–9.8% for girls. Z score exponents of growth by age (z score $> +2$) demonstrated that 6.2–12.7% of boys and 5.9–14.1% of girls were affected (table 1).

Insufficient weight-for-age (z score < -2) was noted in up to 6.6% of boys and girls of all age groups. At the same time, the number of children with excess weight-for-age (z score $> +2$) varied from 1.8 (2–3 months) to 12.0% (12–14 months) for boys, and 1.6 (2–3 months) to 13.4% (15–18 months) for girls (table 1).

Mass growth exponent analysis revealed that the proportion of children with a z score < -2 did not exceed 5.0% in boys or girls of all age groups. The part of children with a z score $> +2$ varied from 7.3 (2–3 months) to 19.1% (15–18 months) among boys, and 6.6 (2–3 months) to 21.9% (15–18 months) among girls (table 1).

All studied exponents of the z score are characterized by increased excess bodyweight (z score $> +2$) with increasing age, with the maximum at 12–15 months. The question whether this fact reflects age-specific physiological characteristics of adipose tissue forming in children, its prognostic value as a risk factor of excess bodyweight in later life and particular qualities of standards of physical development proposed by the WHO requires further study.

Evaluation of energy and nutrient consumption of infants showed (table 2) that almost all children received sufficient amount of proteins, lipids and carbohydrates. Micronutrient consumption was mostly determined by using supplemental feeding product characteristics.

Along with that, our study demonstrated the difference in consumption of nutrients between children who received and did not receive breast milk.

Breastfed children received less proteins, fat (4–6 months), carbohydrates and fiber (table 3) compared with non-breastfed children.

Table 1. Z score levels

Z score exponents	Age						
	2–3 months	4–6 months	7–8 months	9–11 months	12–14 months	15–18 months	19–24 months
<i>Height-for-age</i>							
	Boys						
	n = 55	n = 193	n = 125	n = 175	n = 209	n = 243	n = 246
< -2	14.5	8.3	6.4	4.0	8.6	12.3	10.2
from -2 to -1	21.8	11.9	8.0	9.1	12.0	16.0	11.4
from -1 to +1	31.0	50.8	53.6	59.4	51.2	49.4	54.1
from +1 to +2	20.0	18.1	21.6	18.4	18.2	16.1	15.4
> +2	12.7	10.9	10.4	9.1	10.0	6.2	8.9
	Girls						
	n = 61	n = 136	n = 135	n = 165	n = 169	n = 224	n = 215
< -2	9.8	5.1	3.0	6.6	4.1	8.0	8.4
from -2 to -1	11.5	16.2	11.1	9.7	14.0	12.0	15.3
from -1 to +1	55.7	54.4	49.6	55.8	52.7	54.5	51.2
from +1 to +2	14.8	11.8	22.2	17.0	21.3	15.2	17.2
> +2	8.2	12.5	14.1	10.9	5.9	10.3	7.9
<i>Weight-for-age</i>							
	Boys						
	n = 55	n = 193	n = 125	n = 175	n = 209	n = 243	n = 246
< -2	3.6	0.5	0.8	0.6	1.4	0	3.3
from -2 to -1	16.4	7.8	8.8	2.3	2.9	3.3	7.3
from -1 to +1	56.4	66.8	55.2	56.6	51.2	58.8	51.6
from +1 to +2	21.8	20.2	27.2	29.1	32.5	27.6	27.6
> +2	1.8	4.7	8.0	11.4	12.0	10.3	10.2
	Girls						
	n = 61	N = 136	N = 135	N = 165	N = 169	N = 224	N = 215
< -2	6.6	0.7	0	0.6	0	0.4	2.3
from -2 to -1	3.3	7.4	8.1	2.4	4.1	3.6	2.3
from -1 to +1	68.8	72.1	62.2	60.6	56.8	51.3	61.4
from +1 to +2	19.7	15.4	23.0	27.9	30.2	31.3	23.7
> +2	1.6	4.4	6.7	8.5	8.9	13.4	10.3
<i>Weight-for-height</i>							
	Boys						
	n = 55	n = 193	n = 125	n = 175	n = 209	n = 243	n = 246
< -2	3.6	3.6	3.2	0.6	0.5	0.8	4.9
from -2 to -1	9.1	13.5	8.8	5.7	2.9	0	4.5
from -1 to +1	61.8	52.3	52.0	50.3	47.4	48.5	47.1
from +1 to +2	18.2	18.7	21.6	26.3	30.1	34.2	26.8
> +2	7.3	11.9	14.4	17.1	19.1	16.5	16.7
	Girls						
	n = 61	n = 136	n = 135	n = 165	n = 169	n = 224	n = 215
< -2	4.9	1.5	0	0	1.2	2.2	2.3
from -2 to -1	8.2	8.1	6.7	3.0	4.1	5.4	5.1
from -1 to +1	55.7	59.1	59.2	51.0	46.7	44.6	52.1
from +1 to +2	24.6	20.6	25.2	31.0	33.2	25.9	23.3
> +2	6.6	10.3	8.9	15.0	14.8	21.9	17.2

Figures indicate percentages.

Table 2. Energy and nutrient consumption by children aged 2–11 months

	2–3 months (n = 118)	4–6 months (n = 427)	7–8 months (n = 277)	9–11 months (n = 355)
Nutrients, g/kg bodyweight				
Protein	1.98±0.12	2.30±0.08	2.97±0.09	3.4±0.09
Fat	6.12±0.22	5.04±0.13	4.37±0.09	3.93±0.09
Carbohydrates	11.37±0.50	12.32±0.36	12.75±0.29	13.80±0.32
Fiber	0.15±0.03	0.18±0.01	0.29±0.01	0.30±0.01
Caloric value, kcal/kg bodyweight	108.75±4.20	104.0±2.60	102.36±1.98	104.23±2.13
Minerals, mg/day				
Sodium	225.90±18.14	457.02±24.20	795.92±33.52	1,215.48±38.57
Potassium	592.97±37.83	962.46±30.99	1,399.01±35.79	1,680.83±35.33
Calcium	397.02±31.90	522.32±21.54	626.36±19.97	673.35±19.26
Phosphor	242.82±24.78	394.80±18.79	581.01±19.23	706.86±18.17
Magnesium	42.71±4.58	77.23±3.43	125.84±3.99	155.98±3.71
Iron	2.98±0.49	5.27±0.37	7.19±0.30	8.36±0.32
Vitamins				
A, µg (retinol equiv.)	608.50±56.14	716.04±36.24	707.80±36.00	790.27±78.19
C, mg	60.01±3.36	66.57±2.13	65.53±2.07	61.18±2.04
B ₁ , mg	0.32±0.04	0.42±0.02	0.50±0.02	0.59±0.02
B ₂ , mg	0.77±0.06	0.88±0.03	1.01±0.03	1.10±0.04
PP, mg	2.83±0.31	4.23±0.22	5.74±0.22	6.89±0.25

As the tables show, the caloric value of breastfed infants' nutrition was less compared with the diet of non-breastfed infants; however, the difference was not statistically significant.

Our data are in accord with the results of earlier studies and allow us to conclude the following:

- the incidence of breastfeeding in Russia is insufficient;
- 26% of children start to receive cow's milk and other unadapted cultured milk products from age 4 months;
- specialized manufactured children products are not used often enough in infant nutrition;
- all infants received sufficient amounts of proteins, fats and carbohydrates; micronutrient consumption was significantly determined by the kind of supplemental feeding products used;
- insufficient bodyweight (z score < -2) in boys and girls of all age groups was revealed at an insignificant level of 0.5–5%;
- the percentage of the children with excess bodyweight (z score > +2) was estimated at 1.6–13% and increased with age;

Table 3. Energy and nutrient consumption by children who received (group 1) and did not receive (group 2) breast milk at 2–3 and 4–6 months

	Age 2–3 months			Age 4–6 months		
	Group 1 (n = 84)	Group 2 (n = 34)	p	Group 1 (n = 245)	Group 2 (n = 182)	p
Nutrients, g/kg bodyweight						
Protein	1.55±0.05	3.04±0.35	<0.001	1.63±0.04	3.19±0.17	<0.001
Fat	6.33±0.16	5.59±0.65	>0.05	5.47±0.08	4.46±0.28	<0.01
Carbohydrates	10.77±0.27	12.86±1.59	>0.05	11.01±0.19	14.08±0.78	<0.001
Fiber	0.04±0.02	0.43±0.09	<0.001	0.09±0.01	0.36±0.03	<0.001
Caloric value, kcal/kg bodyweight	106.66±2.57	113.92±13.21	>0.05	100.13±1.32	109.22±5.91	>0.05

- further specification of time of supplemental feeding introduction and range of products and preparation of corresponding recommendations for specialists and parents are needed;
- educational level of the mother plays an important role in establishing the character of infant nutrition;
- doctors and other members of medical personnel do not make enough effort to educate the population about the principles of healthy nutrition.

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Discussion

Dr. Hussain: The reality you describe in your study is similar in all the South Eastern countries, including Bangladesh. Which interventional approach to fight this problem would you suggest to achieve the Millennium Development Goal by 2015?

Dr. Agarwal: We have resources within the country; it's only a question of management and determination to help the vulnerable.

Dr. Stathatos: I would like to ask Prof. Baturin if there are any comparative studies concerning breastfeeding before and after 1990.

Dr. Baturin: About 20 years ago, we had no problems with breastfeeding; about 70% of infants were given breast milk as their main food. But following the introduction of infant formula to the Soviet market, the rate of breastfeeding decreased. After the collapse of the USSR, with the new economic situation and the change in people's mentality, the rate of breastfeeding decreased even further.

Dr. Mohanty: This question is for Prof. Agarwal. The dahi factor in the children's nutrition, was it more due to the animal source plus the probiotic component that it has, was it the combined effect which improved the nutritional status of the children or was it something else? The second question is for Prof. Baturin. After Russia opened up to market economy and the purchasing power of people improved, has this had a direct effect on the nutritional status of children in Russia?

Dr. Baturin: We really have no problem of hunger in Russia, but about 2–3% of children have a low body mass. The percentage of people with a low body mass in the adult population is much greater (3–4%). It's a real problem that we think is connected with low income. We have special surveys dealing with health and nutrition in low-income families, and find that if families have less than maybe 50% of income, they have physical problems, and they have no money to buy food.

Dr. Agarwal: Dr. Mohanty, your question was answered in my study. I compared the milk with the fermented milk and showed a difference, so there is no question whether the probiotic organisms were working or not. They were proteolytic in nature, otherwise these lymphocytic changes wouldn't have occurred.

Dr. Zlotkin: I think if we went around to the representatives of 40 countries, we would see the same general description of breastfeeding in all of our countries. The majority of women breastfeed in the first couple of months of life, but by 6 months of life, the number of women who are exclusively breastfeeding is very low, and I think it's clear that it's not just India, Russia and America, it is most countries. Although I think it's important to identify this as an issue in each of our countries as we do through these surveys, I think the more important issue is to try and identify the reasons why this is the case. For example, in discussions over dinner last night I learned that in the United States the typical maternity leave is 6 weeks and in Canada the maternity leave is one year. It's probably fair to say that if you looked at Nestlé as a company and the women in Nestlé who exclusively breastfeed, it's probably true that those who have a one-year maternity leave breastfeed exclusively for longer than those who have a 6-week maternity leave, and I think you can extrapolate that around the world. I think it's passed the time for us to simply identify this is a problem, and it is the time to start looking at the social determinants of breastfeeding and asking the questions what can really be done about it.

Dr. Agarwal: Maternity leave in India is 6 months, and then the paternity leave can be taken for about 3 months. But the economic factors force the families to work to afford children's education, and education has become very costly.

Dr. Baturin: In Russia, the government supports the cost of giving birth, and the mothers have the possibility to take maternity leave for 3 years. Mothers get full support during 1.5 years, but after that the support is not so big. Our nutrition recommendations and pediatric recommendations are connected with the 6-month duration of breastfeeding, but really I don't understand this problem fully. The mothers don't like this idea, and maybe the problem is connected with our program that gives the possibility of getting food for infants free of charge, including infant formula, milk and kefir. Perhaps this has changed in some regions and they give not only milk but also cereals, but if cow's milk goes to the family, some families use milk for the family, not for the children. In some cases, the mothers change breast milk for milk, and I think it's a problem mostly connected with the education of mothers.

Dr. Villalpando: I wanted to ask Prof. Baturin about anemia. Do you have any data on anemia in children younger than 3 years of age? You mentioned that 50% of the children eat meat in the first year of life, and I think for Mexico that is a lot. We have 2% of wasted children but 40% of anemia at 12 months [1]. So what are the numbers?

Dr. Baturin: We calculated the distribution of anemia among all children. We did not divide children by age, but other data show that about 20% of children younger than 24 months have anemia. We have big regional differences in the distribution of anemia.

Dr. Beard: I have a concern about the problem of anemia that you are presenting, and I am curious if the Russian Pediatric Union has done any mass communication effort to inform parents, in particular mothers, about the dangers of this anemia. Have there been any efforts using mass media such as radio, television or I know you mentioned printed materials for distribution but in newspapers or magazines to inform them of the dangers of using cow's milk so early and encouraging them to continue to breastfeed and to use iron-fortified cereals or other foods as opposed to using the whole milk or cow's milk that may be given to the family?

Dr. Baturin: Really, we use all possibilities to inform parents about anemia prophylaxis, and recommend not to give cow's milk to infants.

Dr. Lack: This is a question for Prof. Agarwal. Part of the big problem in India seems to be the nutritional status of the mothers, and it was a bit depressing to see all statistics on attempts at correcting the anemia. I would like you to sort of speculate as to why that failed. Is this a problem of logistics or compliance, or is there an absorption problem, why can't you correct this very profound anemia?

Dr. Agarwal: In India, there is no shortage of iron folate tablets or syrup for children, but the distribution system fails. It doesn't reach the beneficiaries, and we have no simple answers to this situation at the moment because the country is not governed by laws, rules and regulations, it is governed by individuals [2, 3]. I think the things will change.

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Micronutrient Deficiencies and Effect of Supplements on Correcting Them

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Abstract

The etiology of micronutrient deficiencies in infancy is well described. The deficiencies are caused by one of the following four scenarios: (a) low initial stores of micronutrients from micronutrient deficiency during gestation, premature birth or low birthweight; (b) rapid postnatal growth; (c) ingestion of foods with low concentration of micronutrients, and (d) gastrointestinal pathology resulting in the malabsorption of nutrients, including micronutrients. Understanding the cause of the deficiencies is essential in planning interventions to either prevent or treat them. This chapter will focus on the dietary causes of micronutrient deficiencies and recent strategies to correct them.

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Dietary Origins of Micronutrient Deficiencies

Human breast milk will provide all of the nutrients needed for the otherwise healthy infant during the first 6 months of life [1]. With the exception of vitamin D, this is true. However, it is paradoxical that if dietary variety is the key to the prevention of micronutrient deficiencies, a single food, breast milk, would be recommended as the only source of nutrition in the first 6 months life. However, as is well described in the nutrition literature, if variety is limited, then the quality of the foods eaten becomes even more important in preventing deficiencies. Thus, human milk is of extremely high quality, and this single food meets the needs of rapidly growing infants during the first 6 months of life. However, after 6 months of age, breast milk is not sufficient on its own. There is a need for additional sources of nutrients, especially iron and energy [2]. To meet the energy needs of the infant between 6 and 24 months, there is a need for a source of calories from complementary foods. Similarly,

as illustrated in table 1, there is a need for micronutrients in addition to those found in breast milk and unfortified complementary foods [3]. From this table, it is apparent that the combination of breast milk and unfortified complementary foods is significantly deficient in vitamin A, niacin, vitamin B₆, vitamin D, iron and zinc. The information provided in the table provides a compelling illustration of the rationale for fortification.

Public Health Approaches to the Prevention of Micronutrient Deficiencies

Types of Fortification

There are three types of fortification: general staple food (commodity) fortification, targeted fortification and 'home' or point-of-use fortification. Staple food fortification is the process of adding micronutrients to commodity type foods such as flour, vegetable oil or salt. The major advantage of staple food fortification is that it is inexpensive, and the commodity used is a common food eaten by the majority of the population. Examples of staple food fortification include the fortification of wheat flour with iron and folic acid, cow's milk with vitamins A and D and salt with iodine. The vehicle for the fortificant is generally a staple food eaten by the entire population, including rich and poor, urban and rural. With staple food fortification, the level of fortification must be safe for all consumers of the product, including those who eat the largest amount. Since adult males generally eat the largest amount of food (including staple foods), the level of fortification has to be safe for adult males. By contrast, adult males generally have the lowest requirement for micronutrients. As a result, the concentration of the fortificant in the staple food is often very low. Since children and women (the population at highest risk of micronutrient deficiencies) eat less total food than adult males, staple food fortification is of limit value for women and especially children. Many countries have national legislation for the fortification of the most typical staple foods used in their jurisdiction. In Canada, for example, there is legislation for the mandatory fortification of milk with vitamins A and D, and wheat flour with iron and folic acid [4].

Food fortification plays an important role in ensuring the health of individuals after early childhood. Adding vitamins and minerals to food helps:

- Protect against nutritional deficiencies, for example requiring all milk to be fortified with vitamin D virtually eliminated childhood rickets since the 1970s in Canada;
- Maintain and improve the nutritional quality of the national food supply, for example enriching flour with B vitamins and iron replaces those same nutrients lost in processing;

Table 1. Mean daily nutrient intakes from complementary foods and breast milk compared with recommended intake for 9- to 12-month-old infants living in Bangladesh [3]

Nutrient	Intake from complementary food	Intake from breast milk	Total intake	Recommended intake	Percent of recommended intake
Protein, g	3.6±2.7	7.16±2.0	10.6±2.6	9.6	110
Vitamin A, µg RE	20±49	170±48	191±60	400	48
Folate, µg	16±17	64±18	80±22	80	100
Niacin, mg	1.22±0.91	1.13±0.32	2.36±0.88	4	59
Pantothenic acid, mg	0.36±0.25	1.36±0.38	1.71±0.35	1.8	95
Riboflavin, mg	0.08±0.07	0.26±0.08	0.34±0.08	0.4	85
Thiamin, mg	0.06±0.05	0.16±0.04	0.22±0.06	0.3	73
Vitamin B ₆ , mg	0.08±0.10	0.07±0.02	0.15±0.10	0.3	50
Vitamin B ₁₂ , µg	0.09±0.24	0.73±0.21	0.82±0.27	0.5	164
Vitamin C, mg	4±9	30±9	34±10	30	113
Vitamin D, µg	0.22±0.44	0.42±0.12	0.63±0.40	5	13
Calcium, mg	43±46	211±60	254±54	270	94
Iron, mg	0.65±0.49	0.23±0.06	0.87±0.48	9.3	9
Magnesium, mg	18±13	26±7	44±12	54	81
Phosphorus, mg	63±49	106±30	169±42	400	61
Potassium, mg	120±109	396±112	516±115	700	74
Selenium, µg	11±8	15±5	25±10	10	250
Zinc, mg	0.44±0.30	0.91±0.26	1.34±0.30	2.8	45

- Reduce the risk of diet-related chronic diseases, for example fortification contributes to adequate intakes of calcium and vitamin D which help build strong bones and may reduce the risk of osteoporosis.

In most countries, the addition of vitamins and minerals to food is controlled by Food and Drug Regulations, and only foods fortified with certain nutrients, and to levels specified in the Regulations, may be distributed. For example, in Canada, the current Food and Drug Regulations permit food fortification to:

- replace nutrients lost in the manufacturing process;
- act as a public health intervention;
- ensure the nutritional equivalence of substitute foods, or
- ensure the appropriate vitamin and mineral nutrient composition of foods for special dietary purposes.

Targeted fortification is a proven successful strategy for groups at the highest risk of micronutrient deficiencies. With targeted fortification, the vehicle is specifically a food eaten only by the 'at risk' population. For example, only infants eat infant cereals and infant formulae; thus, for these two foods, the level of fortification is tailored to the specific micronutrient needs of young infants and pre-school children. Since adults generally do not eat infant cereals and formula, there is no concern for use by an inappropriate age group. Most of the iron ingested by infants and young children comes from fortified cereals despite the ready availability of foods containing heme iron, such as meats and poultry [4]. Although targeted fortification is, at least in theory, an efficient method to ensure the micronutrient adequacy of the diet, it assumes that infants are eating foods commercially fortified in the factory where they are cooked and packaged. In most households in developing countries, however, infants do not eat commercially prepared baby foods, but rather eat complementary foods prepared from local commodities and cooked in the home. Similarly, in some households in Western countries, home-prepared baby foods are used in preference to commercially prepared foods. Typical home-prepared porridges are made from rice, wheat, maize or a combination of grains. Although rice, wheat and maize are reasonably high-quality grains, they are low in micronutrients and high in phytate. The phytate binds the micronutrients and inhibits their gut absorption. Thus, on their own, these grains are not good sources of bioavailable micronutrients. The low rates of micronutrient deficiencies in developed countries are to a large degree due to the common practice of feeding infants with commercially fortified cereals. Alternatively, the high rates of micronutrient deficiencies in developing countries are to a large degree due to the common use of unfortified cereals as complementary foods.

From a public health perspective, the use of supplements to prevent micronutrient deficiencies has not been successful with the exception of high-dose vitamin A capsule supplementation to prevent vitamin A deficiency during early infancy.

Globally, it is estimated that 140–250 million children under 5 years of age are affected by vitamin A deficiency [5]. These children suffer a dramatically increased risk of death, blindness and illness, especially from measles and diarrhea [6]. As part of the global call to action, the UN Special Session on Children in 2002 set as one of its goals the elimination of vitamin A deficiency and its consequences by the year 2010. The strategy to achieve this goal is to ensure that young children living in areas where the intake of vitamin A is inadequate receive the vitamin through a combination of breastfeeding, dietary improvement, food fortification, and supplementation.

Combining the administration of vitamin A supplements with immunization is an important part of this effort. Since 1987, WHO has advocated for the routine administration of vitamin A with measles vaccine in countries where vitamin A deficiency is a problem [5]. Many millions of children have been

reached by including vitamin A with National Immunization Days to eradicate polio [7]. Providing immunization-linked high-dose supplementation to new mothers soon after delivery has provided a further benefit to young infants through enriched breast milk. Provision of vitamin A supplements every 4–6 months is an inexpensive, quick, and effective way to improve vitamin A status and save children's lives. The Beaton Report concluded that all-cause mortality among children aged 6–59 months was reduced by 23% through vitamin A supplementation in areas where vitamin A deficiency was a public health problem [8]. However, comprehensive sustainable control of vitamin A deficiency must also include dietary improvement and food fortification.

WHO Perspective

Vitamin A is essential for the functioning of the immune system and the healthy growth and development of children. Immunization contacts offer unrivalled opportunities for delivering vitamin A to children who suffer from deficiency. Studies show that vitamin A does not have any negative effect on seroconversion of childhood vaccines. As well as routine immunization services, national immunization days for polio eradication, measles, and multi-antigen campaigns have been used safely and successfully to provide vitamin A to a wide age range of children at risk (table 2).

In the late 1990s, the global nutrition community was challenged by UNICEF to come up with an alternate solution to targeted fortification that would be applicable to infants and young children in the developing world [9]. Despite isolated successes in many developing countries with general staple food fortification such as with the iodization of salt and targeted supplementation with the use of vitamin A capsules, most countries with high rates of undernutrition were failing to reach malnourished children with effective evidence-based interventions supported by appropriate policies to improve the micronutrient status of children. It is estimated that exclusive breastfeeding and the appropriate use of fortified complementary foods has the potential to reduce mortality among children under 2 years of age by as much as 13 and 6%, respectively. However, commercially prepared fortified baby foods are generally not used in the developing world [10].

The concept of 'home fortification' was introduced and developed by researchers at the Hospital for Sick Children in Toronto, Canada [11]. 'Home' or point of use fortification is a strategy to improve the nutritional quality of home-prepared foods with micronutrient powders containing powdered mineral and vitamin fortificants. For circumstances where the macronutrient and energy density of food provided to children is adequate, but the foods are lacking in micronutrients, micronutrient powder can be added to the food just before it is eaten; thus, the concept of 'home fortification'. The rationale for home fortification was based on the observation that rates of anemia were

Table 2. Potential target groups and immunization contacts in countries with vitamin A deficiency

Target group	Immunization contact	Vitamin A dose
All mothers irrespective of their mode of infant feeding up to 6 weeks postpartum if they have not received vitamin A supplementation after delivery	BCG, OPV-0 or DTP-1 contact up to 6 weeks	200,000 IU
Infants aged 9–11 months	Measles vaccine contact	100,000 IU
Children aged 12 months and older		200,000 IU
Children aged 1–4 years	Booster doses ¹ Special campaigns ¹ Delayed primary immunization doses ¹	200,000 IU

¹ The optimal interval between doses is 4–6 months. A dose should not be given too soon after a previous dose of vitamin A supplement: the minimum recommended interval between doses for the prevention of vitamin A deficiency is one month (the interval can be reduced in order to treat clinical vitamin A deficiency and measles cases).

very low in infants living in developed countries because most commercially prepared foods commonly eaten by infants are highly fortified with iron. Based on the notion that all infants, independent of their socioeconomic status, transition from breast milk to ‘table’ foods by eating semi-liquid complementary foods, it was postulated that ‘home fortification’ could achieve the same results. To accomplish the task of fortifying foods in the home, minerals and vitamins in a powder format are packaged in small single-serving packages (like a sugar sachet) that caregivers could sprinkle over whatever food was prepared for their infant. The advantage of this format is that the powdered minerals and vitamins can be added to any home-prepared complementary food. Thus, there was no need to change traditional feeding practices. To prevent powdered iron from changing the taste or color of the food to which it was added, the iron (ferrous fumarate) is microencapsulated with a thin coating of a vegetable lipid to protect the food from the iron (and the iron from the food) and to ‘taste-mask’ the iron. Thus, microencapsulation prevents any organoleptic changes to the food to which it is added.

Our group has published a summary of the research from six different countries, which demonstrates that the powdered iron in the micronutrient-containing sachets, mixed in maize-, wheat- or rice-based weaning food, is well absorbed, and that cure rates for anemia range from 40 to 90% depending on whether malaria is a predisposing cause of the anemia [10]. A systematic review and meta-analysis of home fortification of complementary foods

was recently completed by Dewey et al. [12] who reviewed the efficacy and effectiveness of home fortification of complementary foods with micronutrient powders (e.g. Sprinkles®) as well as crushable tablets and lipid-based or soy-based products. Sixteen studies (5 anemia treatment trials, 11 prevention trials) met the inclusion criteria for the review. Treatment trials indicate that Sprinkles are as effective as iron drops, are better accepted and have fewer side effects. In prevention trials, the risk of anemia was cut in half. Acceptability of home fortification by caregivers and young children was high, and side effects rare. The authors suggest that the safety of home fortification using 'bolus' doses of iron, particularly in malaria endemic areas, needs further investigation. In one study of Sprinkles in a low-income country, estimates of cost per disability-adjusted life year regained compared favorably with other micronutrient delivery approaches, but the authors of the review indicate that more data on operational and cost considerations for the various home fortification products are still needed [12, 13].

Conclusions

Of the three modes of fortification, general staple food, targeted and home fortification, only the latter two are effective for use in infants and young children. There is evidence that supplements are both efficacious and effective for some vitamins (e.g. vitamin A), but not others (iron). With fortification of staple foods, like wheat flour and salt, the level of fortification is too low for the amount of food eaten by infants and young children. Thus, this mode of fortification is unlikely to be efficacious for this age group. Targeted fortification is efficacious, since the food vehicle and the level of fortification are targeted to infants and young children. However, this mode of fortification is not effective in developing countries because the fortification takes place in the factory where the products are centrally prepared and packaged. Although this type of fortification works well in developed countries, where typically infants are fed with store-bought centrally processed baby foods, in developing countries, where local crops are used to prepare food in the home, targeted fortified foods are simply not available. Most recently, a third type of fortification, home fortification, has been developed. Home fortification has been made possible with the development of micronutrient powders, which are minerals and vitamins in a powder form that are packaged in a small sachet and then added to foods at the point of use. Research studies in developing countries, including Ghana, India, and Bangladesh have proven the efficacy and effectiveness of home fortification as a means to fortify food in the home and to effectively both treat and prevent micronutrient deficiencies.

As a result of these advances, home fortification with micronutrient powders has recently been incorporated as a component of established World Health Organization/UNICEF-recommended feeding strategies [14].

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Discussion

Dr. Haschke: If we look at costs, you mentioned 0.015 dollars (costs of goods) per sachet. We know that the costs of goods are 5–10% of the total costs until the product reaches a person. Therefore, the cost of giving one sachet per day is in the range of 15–20 cents. Do you know the real cost of the project? It is very important whether such a project can be maintained over a long period. It looks quite impressive.

Dr. Zlotkin: The cost that I gave, the one and half cents, is simply the cost of the ingredients, the package and the production process. It did not include any of the downstream costs, and I think multiplying that number by 8 or 10 is probably a reasonable estimate of the total cost. One of the strategies that we have taken around the

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cost issue is to not develop a silo or a vertical program for the distribution of micronutrient powders but to include micronutrient powders in programs that are already in progress. So, for example, UNICEF has programs for the support of breastfeeding and ongoing programs for the appropriate introduction of complementary feeding. So what we have tried to do with my partnership with UNICEF is to include the use of micronutrient powders in the programs that are already paid for by the individual countries. When UNICEF works, it's always with governments, so we try and incorporate the micronutrient powders into ongoing programs. That doesn't negate the total cost of the program but at least spares the necessity to find new dollars for the distribution part. So I totally agree that the cost that I gave you is an underestimate of the total cost, but there are strategies to incorporate those costs into ongoing programs.

Dr. Michel: What can we do in Mexico to get the sprinkles?

Dr. Zlotkin: I am glad you asked that question because in fact my organization has been working with Mexico for the Oportunidades program. Mexico has the dual problem of an increasing rate of obesity in young children and at the same time an increasing rate of micronutrient deficiencies, primarily iron deficiency. The Mexican government has made the decision at least in a number of pilot regions to discontinue or to compare their current strategy, which is giving a fortified milk product to all children, to the use of the micronutrient powders instead, so that they can theoretically not contribute to the problem of obesity and theoretically contribute to the successful prevention or treatment of iron and micronutrient deficiency.

Dr. Klassen: My question concerns consumer understanding. During the last World Congress of Public Health, the World Food Program presented data that were not as convincing as your data because after initial explanation of the program compliance went down to 40–60% depending on the country. The major reasons for this effect were mentioned as: First, a high level of migration led to loss of knowledge of the program. Secondly, the consumers did not understand or misunderstood the package. And lastly, the benefit of taking the supplement was often not understood, since the wording used was not comprehensible to the consumer. Did you do any consumer research to test the understanding of claims related to the supplement?

Dr. Zlotkin: We did some consumer research, but again it was in a control fashion. I think like all programs when they are being delivered to a free living population, when they are true programs, it is really at that time that one has to review the programs, measure things and continue to adapt to the needs of the population. What you say doesn't surprise me at all, and it's not atypical of some of the problems that we have with complementary feeding, even with breastfeeding. There are new children born all the time, and the message that you gave for your first child may have to be repeated it for your second child. The advantage of this intervention is that it is really simple. One doesn't have to be literate to use it, it doesn't change the taste or the color of the food, and it actually works, so I think there are a number of advantages. The disadvantages and especially the disadvantages around iron deficiency anemia is that oftentimes it's very difficult to convince people that their child either has anemia that there is a need to prevent it. I think there must be ongoing education, communication and information in order for these programs to be totally sustainable.

Dr. Kleinman: I noticed as you were talking about Pakistan that the prevalence of anemia decreased from 70 to 30% in children aged under 2 years and then to 20% at 60 months. Does that imply that it's necessary to continue the intervention beyond 5 years of age?

Dr. Zlotkin: Again, to give you a short answer to the question, the anemia rate for under 5 was 56% and 78% for under 2. Of course, we know that the blood volume doubles and triples in the first 2 years of life, and after that growth is pretty slow. So,

between 2 and 5 years of age, the problem of anemia probably is not quite as severe as it is under that. But an ongoing discussion that I have with many of my partners is, who should we target? Should it be children between the age of 6 months and 2 years of age when growth is so rapid, or should it be between 6 months and 59 months of age or 5 years of age? It really depends on the jurisdiction. Some organizations decide that they are going to target all children under the age of 5, others will target the children between 6 months and 2 years of age. It has to do with the amount of funding available, the amount of personnel available and the priorities of the organization.

Dr. Mohanty: What was the impact of parasitic infections, for example hookworm, that cause so many problems?

Dr. Zlotkin: The infants that we included in our studies were generally infants whose average age was 12, 13 or 14 months. Generally, in children under 2 years of age parasite problems do not seem to be a major factor contributing to their anemia. Certainly, above 2 years of age hookworm and other parasitic infections do play a role. So, as you know there are many programs which include the intermittent treatment of parasitic infections above 2 years of age. There are no recommendations for infants under 2 years of age as far as I know. So, although above 2 years it's an important problem, our focus was on infants between 6 and 24 months of age, in which case parasitic infection at least in the younger group does not seem to be a major problem.

Dr. Hussain: I just want to thank and congratulate you and your team for this intervention. After we have adopted it in Bangladesh, our general perception is that it will be as successful in reducing malnutrition as ORS was in reducing diarrhea, and this sachet will definitely contribute to reducing micronutrient deficiency.

Dr. Zlotkin: Thank you. Bangladesh is one of the countries that we did most of the research in. Bangladesh now has probably 5 or 6 distribution models. Distribution has been taken on by the private sector, so it's actually sold in Bangladesh, it's distributed through a number of NGOs, it's distributed through the government and it's distributed through UN agencies. Bangladesh is probably a model country for a multiple distribution methodology, and you need that in order to have good enough coverage.

Dr. Lack: You had your strategy slide and discounted a number of strategies. I note you didn't include GMO crops, and a while ago there was a lot of talk about vitamin A introduction into rice. I just wondered whether that was a strategy that you thought might have any value. Of course, it's very convenient if you can just eat your local food and it provides everything you need, or do you think that's sort of science fiction?

Dr. Zlotkin: I am not aware of universal success or national programs which include vitamin A golden rice, but I think the idea is a reasonable one. I think there are many fabulous ideas available. In fact, if you look at the *Lancet* series from 2 years ago on the prevention of child mortality, there are probably 20 or 22 evidence-based interventions that are known to work, that if they were implemented, it would decrease the rates of infant and child mortality in the world by probably 80%. So, if I were to give advice to young scientists in the nutrition field on the area that they should work in in order to have the biggest impact, I would say it would be in the new field of what people are calling implementation science. It is a science or should be a science, and I think that's where the money should be spent.

Dr. Siega-Riz: I was wondering, what you have actually dealt with is developing a product that helps us improve the micronutrient deficiencies. However, this doesn't actually address the issue of additional calories for the wasted or stunted child. I was wondering if you could talk a little bit about whether or not you actually see beneficial effects on growth perhaps.

Dr. Zlotkin: We don't see effects on growth, and you are absolutely right, the micronutrient powders contain no source of energy, no sources of protein, and in fact these are only micronutrient supplements. They do not solve the problem of global

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undernutrition, where one assumes that it's a combination of inadequate energy intake as well as inadequate micronutrient intake. Micronutrient powders are not going to solve all the problems of malnutrition. As you know, there are some other innovative interventions that are used for children with severe malnutrition and starvation, including the ready to use foods, that have been very successful.

Dr. Stettler: When you see such high rates of iron deficiency and it's so hard to address, one wonders whether there is some type of evolutionary benefit to it and how human mankind evolved with such a high rate. I wonder what your thoughts are about that and if we are really successful in addressing iron deficiency. Are there going to be negative health consequences that we may not have thought about?

Dr. Zlotkin: The last question was, are there negative consequences of action preventing it?

Dr. Stettler: I mean if there is an evolutionary benefit to iron deficiency, if you are able to address it successfully like you seem to be addressing it, might there be any possible negative impact?

Dr. Zlotkin: Just very briefly, one of the concepts is that when we became an agrarian society, we actually changed our diet which was primarily based on meat or fish to a diet that was based on wheat and other crops that we could grow. There are a number of people who talk about the evolutionary consequences of changes in diet. I think iron is certainly needed by the human; it's also needed by the organisms that live with us, including the organisms that make us sick, and the best example of that is malaria. Researchers are asking whether or not there is a negative impact of providing iron to children with iron deficiency in a malaria-endemic area. There are a number of research projects now which are studying that question. The theory is that the malaria parasite uses the iron somehow or it has an effect on the immune system such that a child who has a better iron status is actually less able to cope with the malaria parasite than a child whose iron status is poor. So, I think the issue is a true issue but actually too big for a brief discussion right now.

Dr. Lake: In one of your early slides where you looked at the prevalence of iron deficiency by country, I was struck by how low the prevalence appears to be in China. What is China doing right?

Dr. Zlotkin: We have done actually two studies on iron, and I worked with the Chinese CDC, which is the equivalent of the American CDC in Atlanta. We went to the first area which was in Inner Mongolia. We started the study, and we could not find any children with anemia. So the CDC said, 'no problem, we'll go to a different area'. We went to another rural area, and once again although I was told that the rates would be in the range of 30–40%, the ranges were actually less than 10%. I don't know exactly what it is that they are doing in China to change the rates from what they were probably 50 years ago, but the slide that I showed on poverty is probably the right answer. I think China is a great example of a country in transition from a very underdeveloped country to a developed country, although it is still in transition. When we talk about China, there is western China which is poor, there is Shanghai that is rich, so there is no such thing as one China, but I think that the answer is a combination of improved economics in the family, improved hygiene, improved food, having bathtubs in the house, watching television, etc. I think it's a combination of effects that is changing the life and the health of Chinese.

Dr. Stoll: I have a question concerning safe intake. When you provide the treatment for a month are you not afraid of overdosing, especially zinc and vitamin A?

Dr. Zlotkin: We addressed that in a number of ways. The first way was that the micronutrients are in a powder and part of that powder is what is called an excipient or a filler. Originally, we thought it would be a good idea to use sugar as the filler because then it would be easy to get the child to take the micronutrient powders. We

quickly decided that that would not be a good idea, so the filler we use is a malto-dextrin, which really isn't sweet at all, it has a very neutral taste. If you were to taste the powder, it tastes slightly sour from the ascorbic acid, but otherwise it has a very neutral taste. So there is no real reason why an older child in the family would open a package and want to eat more than one package because they simply don't have a good taste. In addition, although it's easy for a parent to open the package, it's a 3 layer package with paper, polyethylene and foil or aluminum, a young child would have a hard time opening it. And the final thing is, because the amount of iron in the package is approximately 10 or 12.5 mg, in order to approach the amount of iron that would be lethal, a child would have to eat something like 16 or 17 packages. So, the combination of having a neutral taste, the package that is not too easy to open but not too hard to open, and having to use 16 or 17 packages before toxicity would occur we think addresses the issue of safety and in all of the many studies that we have done, including many of the distribution programs, we have not heard anyone who has actually used more than one or two packages a day.

Dr. Stoll: I was not concerned about iron but mainly zinc. You mentioned that you are adding 5 mg of zinc per sachet, and you can easily overdose if you have 2 or 3 sachets per day.

Dr. Zlotkin: Good point.

Dr. Lack: Just getting back to the evolutionary question and the comments made about China, I wondered whether there are any epidemiological data on migrant ethnic communities say in the US comparing rates of anemia in Chinese, black African, Hispanic communities and correcting for demographic factors to see whether there might be sort of genetic tendencies.

Dr. Zlotkin: That's a great question, and maybe someone in the audience knows the answer. I actually don't know whether that has been done. Does anyone else know?

Dr. Kleinman: In the US, WIC covers a very large proportion of young infants, and those who are in need of support are given foods and supplements that would reduce the risk of anemia. In fact, the WIC program has reduced the prevalence of anemia significantly in the low-income population in the US. I think one evolutionary issue that you didn't bring up is that for most of human history, infants crawled in the dirt, and the soil is a fairly rich source of iron that isn't available anymore to most babies, particularly in our hyper-hygienic society.

Dr. Stettler: I have a comment, not a question. I really like the concept that you tested about flexibility, that it's OK if you miss one day. I think this is really something that should be an inspiration for those of us who work in a wealthy country on chronic disease, obesity, and cardiovascular prevention. Our messages so far have been pretty rigid: 5 servings of fruit and vegetables every day, physical activity every day. I think if we were able to give the people some flexibility and permit them to miss one day once a while, this would be a really nice approach. I thought that was really inspiring for domains other than the one you are working on.

Dr. Ganguly: Is there any form of nutrition counseling which is being given along with the distribution of these sachets to make a positive impact on the dietary habits?

Dr. Zlotkin: As I mentioned earlier, we try not to place the micronutrient powders into a silo program, which is a program that specifically and only addresses micronutrient deficiencies. In working with government organizations, non-government organizations and the UN organizations, we try to include the use of micronutrient powders with on complementary feeding and general feeding advice. So, what we might say is that 6 months of age is an appropriate time to introduce complementary foods. In order to increase the nutritional value of the complementary foods we suggest that you use one sachet or 30 sachets over the next 2 months, and in the sachets are those ingredients which you might not be able to get from your local food. We

would also give advice on most appropriate local foods to use, so the answer is yes, we try and include the concept of home fortification with other horizontal programs as opposed to including new vertical programs for the appropriate age groups. The only time when that might not be done is when the private sector is involved in distributing the sachets. There, we have little control over what information is provided.

Dr. Agarwal: For children, we should not fix any age when to give iron. The situation varies from country to country. For a country like India, pregnancy anemia and lactation anemia remains the number one priority because it is in the later part of pregnancy that the iron is transferred and needed for neurotransmitter formation. Transfer of iron through placenta is reduced as we have showed in our studies [1–9]. I think we should not make any recommendation as to what should be the age to supplement iron.

And point two; you said that the wheat flour should not be fortified because adults eat it. How will a pregnant woman get it, how will a lactating woman get it? In India, the sprinkles are supplied but the national level of iron is zero, 100% have iron anemia, and therefore in this nation iron in any form is essential.

Dr. Zlotkin: I totally agree, and I absolutely think that large commodity should be fortified, and they are of great value to adults who eat large amounts of those fortified foods. My point was that food fortification is important but will not impact very much on the young child. I hope I didn't say that we should get rid of large commodity food fortification. I didn't mean that, I just simply meant that it doesn't work for very young children.

Dr. Jones: The salt problem with iodine has been an issue in Tasmania. We are mildly iodine deficient, but the recommendations are to limit salt because of blood pressure and other issues. So we put iodized salt in bread, and that actually works at the population level. Also, did you look at how much vitamin C should be taken, because obviously vitamin C has a major effect on iron absorption?

Dr. Zlotkin: We actually did some research looking at a dose-response for vitamin C in the sachets and how much iron would be absorbed using our stable isotope methodology. We found that between 30 and 50 mg was the right amount. The other important issue was the issue of practicality. If we used more than 50 mg, the sour taste of the vitamin C actually changed the taste of the food into which it was added.

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Food Allergy and Complementary Feeding

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Abstract

The relationship between complementary feeding and the development of atopic disease is the source of significant interest and debate in both the scientific and lay communities. A small number of early studies, which had considerable influence on recommended feeding practices, reported protective effects associated with delaying the introduction of commonly allergenic foods such as cow's milk, egg, and nuts. Despite more conservative recommendations, however, food allergy prevalence has continued to rise. Our understanding of the development of food allergy, its relationship with IgE sensitization and atopic dermatitis, and the relationship of each of these outcomes with the timing of food introduction has evolved considerably. Based on multiple observational studies, and extrapolating from immunotherapy trials and animal models of mucosal immunity, there is mounting evidence that delayed introduction or avoidance of commonly allergenic foods is at best neutral and may be detrimental with regard to atopic outcomes. There is an obvious and critical need for additional high-caliber studies to further evaluate this connection. In the meantime, multiple health considerations, not allergy alone, should be involved in decisions regarding nutritional intake, including common allergenic foods, during the period of transition to the family diet.

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Introduction

Complementary feeding refers to the introduction of first foods and the transition from breast milk or infant formula to the adoption of the family diet, usually completed by around 18–24 months of age. This is accompanied by decreased reliance on liquid as a primary source of nutrition.

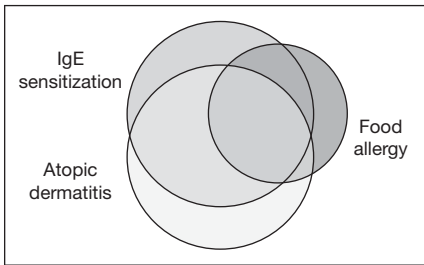


Fig. 1. Model of the overlapping but distinct outcomes discussed.

Atopic diseases have increased rapidly and in correlation with westernized lifestyle, strongly implicating environmental factors. Clinical food allergy, sensitization to foods (positive skin or serum testing), and atopic dermatitis often occur within the first 1–3 years of life. A few foods (milk, egg, wheat, peanut, soy, fish) account for a large percentage of food allergy in young children.

Some influential early studies found evidence that delaying introduction of more allergenic foods was potentially protective against food allergy and/or atopic dermatitis at an early age [1–3]. Based on these findings, both US and European bodies endorsed conservative recommendations or policy statements with respect to the timing of introduction of some foods [4, 5].

Despite these recommendations, the incidence of food allergy and atopic dermatitis has continued to rapidly increase over the past decade – by some assessments even faster than that of other atopic disease (e.g. asthma, allergic rhinitis) [6, 7]. In addition, the preponderance of data now available fail to show clear risk reductions for these outcomes by delaying the introduction of foods [8], and some suggest the opposite: that delayed introduction may enhance risk of food allergy [9–14]. However, the data are still limited and sometimes conflicting, especially given that clinical outcomes, such as food allergy, are often only partially characterized by measuring specific IgE.

In order to relate this literature to the questions most often posed by patients and their families, it may be helpful to separately evaluate what is known about the effects of complementary feeding on clinical outcomes in high- or low-risk infants. Furthermore, this must be achieved with the understanding that there is overlap, but not tight correspondence, between these outcomes (fig. 1). Those outcomes that are of most interest to patients are clinical food allergy (IgE mediated or non-IgE mediated), atopic dermatitis and the course of established allergy. Allergic sensitization (positive skin prick or serum IgE test) is not a clinically relevant outcome per se. When present, it should be rigorously corroborated with the clinical history, ingestion challenges and periodically reassessed. This is especially true early in life

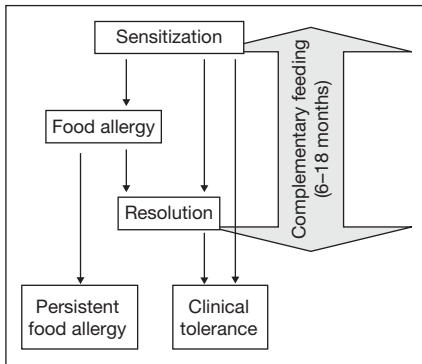


Fig. 2. Model of the development or resolution of food allergy relative to the weaning period.

as the relationships between exposure, sensitization and clinical disease are often very dynamic over time (fig. 2).

As the available data are often insufficient for making recommendations that are truly evidence based, until prospective randomized controlled studies are designed to address those gaps, we must cautiously use the information we can deduce from other contexts including observational studies, immunotherapy trials, and even animal models of mucosal immunity.

Modifying the Risk of IgE Sensitization

Allergic sensitization refers to the documentation of allergen-specific IgE, either by in vivo skin prick testing or detection of specific IgE in serum. Two things are important to note about this outcome. First, there can be biologically relevant discrepancy between skin prick testing and in vitro serum testing. Skin testing is an in vivo exposure, and reactivity depends on the presence of allergen-specific IgE with the capacity to induce cross-linking of high-affinity IgE receptors on mast cells armed with allergen-specific IgE – a capacity that is influenced by qualities of the IgE repertoire [15] (e.g. the polyclonality, the individual affinities of those clones, and the specific/total IgE ratio) as well as the intrinsic properties of the patient's mast cells [16]. Second, regardless of the method of detecting sensitization, only a subset of those who are sensitized will prove to be clinically allergic. For example, while the prevalence of clinical peanut allergy is approximately 0.5–1% [7], a large unselected population-based evaluation of peanut sensitization by skin testing revealed a prevalence of 8.6% [17], suggesting a false positive rate of ~90%. This rate is likely to be lower among younger children who have not

yet become sensitized to potentially cross-reactive airborne allergens [18]; however, in a study of 1,072 children 4–5 years of age, the false positive rate was still ~35% [19].

One of the early studies to address the role of complementary feeding in high-risk infants did find evidence of protection and played a significant role in supporting conservative recommendations of potential allergen exposure [3]. Zeiger et al. [20] conducted a prospective, randomized, interventional study of high-risk children, where either the introduction of common allergens (milk after 1 year, egg after 2 years, peanut after 3 years) was delayed, or standard feeding practices were followed. The intervention also included maternal avoidance of common allergens in the third trimester and while breastfeeding. Families following the conservative schedule had lower rates of sensitization to milk at 24 months of age. However, in a follow-up report at 7 years of age [3], no significant difference in sensitization to any food allergen remained.

A systematic Cochrane review on the optimal duration of exclusive breastfeeding published in 2002, and updated in 2009, did not assess the outcome of food allergen sensitization [8]. However, Zutavern et al. [21] reported the rate of sensitization to foods (egg, milk, peanut, soy, wheat and fish) by serum IgE testing (CAP-FEIA) in a large birth cohort. Feeding was assessed at 6 months. Approximately 1/3 of the children received foods before 4 months of age, while half were exclusively breastfed in the same period. Twelve percent of the analyzed population were sensitized to one or more of those six foods. To take into account reverse causality, analyses were also performed excluding children with rash or allergic symptoms in the first 6 months of life. There was no protective effect of a late introduction of solid foods (>4 or >6 months) or a less diverse diet within the first 4 months. In contrast, sensitization was significantly more common with late food introduction: adjusted odds ratios for no solid food introductions until 4–6 months or >6 months of age were ~3. Similarly, a Finnish cohort of almost 1,000 infants examined the prevalence of sensitization to both inhalant and food allergens, and found increased rates in children with delayed food introduction [22]. Curiously, given some paradigms of oral tolerance, this did not appear to be an antigen-specific, but rather a generalized effect. Finally, the Australian Childhood Asthma Prevention Study, including ~500 healthy infants, also failed to demonstrate a protective effect of late complementary feeding on allergic sensitization, though no benefit was seen either [23].

In contrast, though not directly bearing on the question of infant solid food introduction, a very recent paper on the role of peanut consumption during pregnancy and breastfeeding deserves mention. As part of the Consortium for Food Allergy Research, a cohort of individuals (3–15 months) at high risk for peanut allergy by virtue of established milk or egg allergy was collected by Sicherer et al. [24]. More than 25% of these young children had peanut-specific IgE levels of 5 kU/l or higher – a level that the authors considered as

'likely indicative of peanut allergy'. Furthermore, they found by multivariate analyses that maternal ingestion of peanut during pregnancy was associated with this level of sensitization (OR, 4.99; 95% CI 1.69–14.74; $p < 0.004$). It must be emphasized, however, that clinical allergy was not assessed. It may well be, in fact, that among high-risk populations, sensitization is very common and widely discrepant from clinical allergy as discussed above. As an additional illustration of this likely pitfall, approximately 40% of high-risk infants at 4 months of age enrolled in a randomized controlled trial of peanut flour introduction [25] (discussed further below) are sensitized, yet the large majority are tolerating regular ingestion [Lack, pers. commun.].

Modifying the Risk of Atopic Eczema

A New Zealand cohort study evaluated the relationship between eczema and the diversity of solid foods introduced in the first 4 months of life. The rate of recurrent or chronic eczema was high (7.5%). No information was given on allergic sensitization to foods or on other allergic disease including food allergy. They reported a positive dose relationship between the diversity of foods introduced before 4 months and the risk of eczema at 2 and 10 years [26]. They were unable to link a significant risk to a specific food (milk, egg, cereals, vegetables, fruits).

In contrast, when examining the effects of delayed introduction of complementary foods on risk of eczema, most studies report no benefit or increased risk. The Cochrane review on the optimal duration of breastfeeding identifies two qualified studies that addressed atopic eczema in the first 12 months ($n = 3,618$) and one at 5 years of age ($n = 113$) [8]. The combined analysis failed to find evidence of protection at either time point. Along with the absence of effect of delayed feeding on allergic sensitization mentioned above, the Australian Childhood Asthma Prevention Study also failed to reveal a benefit for eczema [23].

As with the risk of allergic sensitization, some studies have reported lower rather than higher risks of eczema associated with earlier food introduction. For example, in a Swedish birth cohort, regular fish ingestion before 12 months was associated with lower rates of atopic disease, including eczema, at 4 years [11]. And an increased risk of eczema was also associated with delayed solid food introduction in the German cohort discussed above [27].

Modifying the Risk of Clinical Allergy

Most cohort studies evaluating the role of environmental factors in the development of food allergy, including feeding practices, have relied on reported history without confirmatory food challenges or markers of strong

sensitization with generally accepted predictive value, and so are likely to overestimate the prevalence of disease. Furthermore, many studies do not distinguish between IgE- and non-IgE-mediated disease.

In their first report of high-risk infants participating in an interventional study including both maternal avoidance (third trimester and during lactation) and delayed introduction of common allergens, Zeiger et al. [20] reported a 3-fold higher combined rate of 'food-associated atopic dermatitis, urticaria and/or gastrointestinal disease' at 12 months in children following a standard schedule of solid food introduction. However, at 7 years of age, there were no differences in reported food allergy between these groups [3]. Another early study of a small Finnish cohort ($n = 135$) also found a potentially transient benefit of delayed solid foods: self-reported food allergy at 1 year was significantly lower. However, the benefit was not significant when food allergy was defined by challenge, and was not found at the 5-year follow-up [2, 8].

Several influential thought leaders raised persistent and early objections against the trend toward more conservative feeding practice recommendations [28–30], but particularly influential data have come from the comparisons of Israeli and UK Jewish populations with widely discrepant practices of peanut introduction and prevalence of peanut allergy, despite very similar rates of atopy. du Toit et al. [10] reported on approximately 10,000 children divided between the two countries and found that while the prevalence of peanut allergy in the UK group was 1.85%, the prevalence in Israel was 0.17%. The adjusted risk ratio accounting for atopy was 9.8. There was a striking inverse correlation with the early ingestion of peanut protein between the two populations, which was a median 7.1 g/ month (frequency 8×/month) in Israeli infants between 8 and 14 months and 0 g/month in the UK. Largely on the strength of this observation, a large randomized interventional study introducing peanut flour in high-risk infants is underway [25].

Further supporting the potential benefit of early allergen exposure, two very recent prospective observational studies have reported that earlier introduction of cow's milk protein (CMP) [13] and egg [14] is protective against IgE-mediated allergy. In their prospective observational study of 13,000 infants, Katz et al. [13], found that the mean age of CMP introduction was significantly different ($p < 0.001$) between the healthy infants (61.6 ± 92.5 days) and those with IgE-mediated cow's milk allergy (116.1 ± 64.9 days). Only 0.05% of the infants who were started on regular CMP formula within the first 14 days versus 1.75% who were started on formula between the ages of 105 and 194 days had IgE-mediated allergy ($p < 0.001$). The odds ratio was 19.3 (95% CI 6.0–62.1) for development of IgE-mediated milk allergy among infants with exposure to CMP at the age of 15 days or more ($p < 0.001$). Similarly, Koplin et al. [14] found in their study of 2,589 infants, that introduction of egg at 10–12 or >12 months was associated with increased risk (OR 1.6 and 3.4, respectively) compared with introduction between 4 and 6 months. This risk was found in both high- and low-risk infants and persisted after correction for multiple potential confounders.

Based on these accumulating data, several official recommendations and position statements have changed to reflect the shifting consensus. For example, the American Academy of Pediatrics, which in 2000 endorsed delayed introduction of egg, peanut, tree nuts and fish, now concludes, 'there is no current convincing evidence that delaying solid food introduction beyond this period has a significant protective effect on the development of atopic disease. . . This includes . . . foods that are considered to be highly allergic, such as fish, eggs and foods containing peanut protein' [31]. The ESPGHAN goes somewhat further recommending against both early (<4 months) and late (>7 months) introduction of complementary foods without respect to potential allergenicity [32].

Modifying the Course of Established Allergic Disease

A related but distinct question is what role dietary allergen exposure may have in modifying established clinical disease. This has been addressed largely outside the time of complementary feeding, in older children with persistent egg or milk allergy who have already transitioned to the family diet. For example, Allen et al. [33] surveyed approximately 200 families of older children (mean age 6.6 years) with egg allergy and concluded that strict avoidance of egg and accidental ingestion of egg did not appear to influence the acquisition of tolerance. Studies have shown that a substantial proportion (~2/3) of children with milk or egg allergy tolerate immunologically significant amounts of allergen in other foods – particularly when they have been extensively heated [34, 35]. Intentional exposure of these children to allergen in this form has been associated with immune responses that correlate with tolerance [34–36]; however, a randomized study to address this has not been reported. The immunological effects of oral exposure in those with established disease receiving oral immunotherapy may substantially overlap with those that occur as a result of more casual exposure. However, given the capacity of IgE to facilitate antigen presentation and influence adaptive immune responses, the exposure of allergen in an individual with high-affinity IgE may have significantly different effects on specific immunity [37]. In addition, because infancy is likely to be a critical period for establishing oral tolerance, earlier interventions in allergic individuals may be warranted regardless of findings in older children.

Conclusions

Food allergy is thought to be a manifestation of failed oral tolerance induction that is the result of complex interactions between gut permeability/maturity, bacterial colonization, and the timing of antigen exposure.

Complementary feeding recommendations must be informed by multiple health considerations of the infant/toddler transitioning from breast milk (or infant formula) to family food. Though randomized clinical trials are needed, the current data generally point to an increased risk of food allergy or related conditions associated with delayed introduction of solid foods, including those regarded as more allergenic such as peanut, egg and fish and regardless of infant risk. This tentative conclusion is also more consistent with current paradigms of oral immune tolerance.

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Discussion

Dr. Fasano: I think that you did a great job to give us a 'twenty thousand feet up in the air' overview, and I want to make sure that you expand some very important messages that you just conveyed for us. I think that there is no debate anymore that immune progression from tolerance to immune response is a generalized machinery that applies to allergy, autoimmune diseases, inflammation in general and also the concept that the final destination does not always match with clinical outcome. I think the most intriguing message that you just gave us is that in a situation of food allergy like cow's milk protein intolerance, which we know that the vast majority of kids grow out of within a certain period of time, there are two events that seem to be really decisive in switching from a new response to tolerance. If I got the story right, basophils would go down and the T reg would go up, and these are your biomarkers. It would be tremendously helpful to understand what kind of tricks they used to outgrow this, because the same tricks can then be applied to kids that do not outgrow for example peanuts or shellfish allergies. And to my second question: cow's milk protein intolerance, it's something that really is not tissue specific; you start the game at the level of the intestine, but the skin can be involved as a clinical outcome, the airways can be involved, within the GI tract you can have the colon involved, but not the small intestine and vice versa. What is the trophism in terms of tissue targeting that dictates the clinical outcome? Do we know anything about that?

Dr. Shreffler: Regarding the first question, the basophil sensitivity to milk among those children who are more tolerant -- prior to a kind of natural high dose exposure -- is lower, probably purely reflecting differences in their IgE repertoire. It's then further suppressed with active allergen exposure -- this is what we've published in the context of the milk allergic children who outgrow their allergy [1]. At the same time, yes, these patients also have higher frequencies of specific Tregs, and one of the mysteries in this is how is that regulatory T cell population expanding in the absence of intentional exposure, because that study is not OIT, that's just natural progression [2].

The antigen exposure necessary to expand regulatory T cells or -- when things go wrong -- expand the effector T cells, is probably explained by low antigen exposure to these ubiquitous antigens occurring from various routes, including skin, respiratory and occasional ingestions, etc. Sampling of dust from households and public environments reveals the presence of low levels of allergen in the environment, and I think this is likely even in the families that are doing their very best to avoid the allergen.

Why this doesn't happen as readily for that individual sensitized to peanut or shellfish or something that's associated with more persistent disease, or indeed why it doesn't happen in the subset of milk-allergic children who have persistent disease, I suspect may be due to differences in that initial sensitization period -- that the IgE repertoire from the beginning is of higher affinity, is more T cell dependent, is more polyclonal, and that when that occurs it sets up a milieu that is more difficult for tolerance to then reassert itself. That would at least be consistent with the observation that allergy to nut and shellfish does tend to be more severe, and in those children with milk allergy who don't outgrow persistence and clinical sensitivity at least weakly associate. While any child with milk allergy, even a child who outgrows by 3 or 4 years of age, may certainly have a severe reaction, on the whole these phenotypes track together, and those kids that don't outgrow their milk allergy well into adulthood are the ones that scare us because they have very high specific to total milk specific IgEs, they have very high-affinity IgE, they have severe reactions. I think that with that kind of early insult, whatever it was, and the establishment of a robust IgE response probably pretty early on, the odds are against them from that point forward for tolerance to reassert itself. And I think in our efforts to intentionally induce tolerance, this may

well be a substantial obstacle for us as well. But of course that's what we hope to find out more about.

Dr. Fasano: What about the tissue trophism?

Dr. Shreffler: I don't think all that much is known. There is evidence of some shared T cell homing markers between gut and skin, and that T cells primed in one location are able to home to either site and that that might be physiologically very important. I am particularly interested in the skin route of sensitization vis à vis this retinoic acid story which I didn't go into at all, but we have evidence that a protein in peanut can directly induce RA production. Perhaps outside of the gut -- which is generally already RA rich -- maybe that's particularly sensitizing rather than tolerogenic. Whether or not it's allergenic, however, it should at least be inducing gut trophism. I guess one other point is that I showed you that while the basophil suppression is enhanced by antigen exposure, the T regulatory cells actually disappear from the peripheral blood once these kids start eating antigen, and so our hypothesis about that is that they probably go into the gut, but of course maybe they are apoptosing or something else.

Dr. Lake: Would you want to comment on intrauterine sensitization?

Dr. Shreffler: One can measure allergen-specific IgE in cord blood, and certainly we can measure IgE very early in life. I showed that paradigm of TH2 and IgE, and I presented IgE induction as a T-dependent process. It's very clear, however, and a good example of this is in some immunodeficiencies, that there are non-T-dependent pathways of IgE induction, and these are probably relevant outside of the immune deficiency as well. For example, the skin of kids with atopic dermatitis pumps out lots of BAFF, a factor that's known to be sufficient to induce IgE class switching in B cells in a T-independent fashion. This suggests the hypothesis that some of the IgE that we measure early in life are not secondary to antigen exposure via the maternal bloodstream followed by an adaptive immune response in the baby. Certainly one of the hallmarks of this sort of non-T-dependent IgE repertoire would be that it would tend to have low affinity and be expressed from germline sequence without somatic mutation akin to 'natural' IgM antibody. Allergens are often decorated with the sort of non-mammalian glycans that natural antibody may recognize with low affinity. So, to what extent the IgE that we measure very early in life or from cord blood is truly reflective of a T-dependent, antigen-dependent process, I am not sure.

Dr. Saavedra: When you presented the data on IgE repertoire, there were various proteins that you showed in terms of potential for outgrowing. I know there is a large number of casein proteins and a relatively small number of whey proteins. Would you care to comment on the difference between casein sensitization and tolerance versus whey?

Dr. Shreffler: I think there are pretty convincing data that whey and casein fraction proteins are handled differently by the immune system. Casein is handled like particulate allergen via Peyer's patch; casein allergens are very important sensitizers. Whey proteins, in contrast, are much more readily absorbed through the epithelium. My colleague Cecilia Berin at Mount Sinai had some very nice work together with Lloyd Mayer showing in part that the sensitization to whey proteins in an animal model might play a larger role in anaphylaxis because they are rapidly absorbed into the bloodstream [3].

There is a body of literature comparing casein and whey sensitization with respect to clinical outcomes in humans.

Dr. Mohanty: Clinically, what we see is once children developed allergy (for example cow's milk allergy), they grow out of this allergy as the age advances. So first there is tolerance, then there is allergic manifestation, i.e. the disease, and then they grow out of it, meaning they probably developed tolerance again. In your model, is there something which can explain this?

Dr. Shreffler: I don't know about the early tolerance and then disease and then tolerance again. I think what's truer to my experience is that they may well have been getting milk in their diet but probably had some at least suspected pathology that in retrospect was probably real, such as bad eczema and you took the milk out of the diet and the eczema resolved, or much less frequently, in my experience, perhaps some chronic respiratory disease. You are saying that you take the milk out of their diet and they get better, and then they ultimately outgrow the allergy altogether. I think that is similar to the patients I am describing who pass a challenge to heated milk and once that is added to the diet and they actually get regular exposure to milk it seems to be beneficial.

Dr. Mohanty: If there is a child with a family history of allergic diathesis, instead of introducing cow's milk as a complementary food during the weaning time, can we test his/her IgE level, then give cow's milk for some time and then repeat the IgE test? If the IgE level is very high, we could then stop giving cow's milk even though the child has not developed symptoms of allergy.

Dr. Shreffler: I think that if they are tolerating cow's milk in the absence of symptoms regardless of the IgE, I would be loath to take it out of the diet at all. But if they had disease and they have IgE -- a disease that you were convinced was provoked by milk -- and now they are able to tolerate some milk protein, then I think following IgE over time and using that as a guide to expand the milk in the diet is a reasonable approach.

Dr. Jones: We have a long-term cohort, a 16-year birth cohort, where we have looked at sensitization in utero. We don't find anything predicts cow's milk or egg allergy, but peanut allergy is in fact predicted by peanut intake in the mother independent of the child intake during childhood and it's actually modified by the family history of atopy, so the question over there is relevant. If there is a family history of atopy, the peanut intake during pregnancy is a strong positive predictor of peanut allergy at 16, but if there is no family history of atopy, avoiding peanuts during pregnancy actually increases your risk 5-fold. For ryegrass, we actually don't find in utero exposures have any role, but season of birth does. So if you are born during the high ryegrass period in Tasmania and get a viral infection at that time, you have a very high risk of having RAST-positive ryegrass testing

Dr. Shreffler: Yes, it's interesting. Scott Sicherer has a high-risk cohort of much younger patients, obviously not 16 years of follow-up, but also found evidence of more IgE sensitization with maternal exposure. The problem with that is that maternal exposure doesn't necessarily mean that the relevant route was the mother's ingestion as there might be more peanut in the home, etc.

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Early Feeding: Setting the Stage for Healthy Eating Habits

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Abstract

Food habits, an integral part of all cultures, have their beginnings during early life. This chapter reviews the development of the senses of taste and smell, which provide information on the flavor of foods, and discusses how children's innate predispositions interact with early-life feeding experiences to form dietary preferences and habits. Young children show heightened preferences for foods that taste sweet and salty and rejection of that which tastes bitter. These innate responses are salient during development since they likely evolved to encourage children to ingest that which is beneficial, containing needed calories or minerals, and to reject that which is harmful. Early childhood is also characterized by plasticity, partially evidenced by a sensitive period during early life when infants exhibit heightened acceptance of the flavors experienced in amniotic fluid and breast milk. While learning also occurs with flavors found in formulae, it is likely that this sensitive period formed to facilitate acceptance of and attraction to the flavors of foods eaten by the mother. A basic understanding of the development and functioning of the chemical senses during early childhood may assist in forming evidence-based strategies to improve children's diets.

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Introduction

The unhealthy eating habits that plague adults – too many calories and salty, sweet, and fatty foods, too few fruits and vegetables – are also rampant in the youngest members of society. Infants and toddlers consume an estimated 10–31% more energy than recommended [1], but not by overconsumption of fruits, vegetables, whole grains, or lean proteins – French fries are the 'vegetable' they most commonly consume [2, 3]. Eighteen to 33% of infants and toddlers consume no servings of vegetables on a given day, and 23–33% consume no servings of fruits [2]. Additionally, almost half of infants and toddlers consume desserts, sweets, or sweetened beverages daily [2, 3].

The negative impact of these dietary patterns manifests in increasing obesity among children, a worldwide public health crisis [4–8]. Health professionals recommend that children reduce intakes of added sugars, sodium, and saturated fats and increase intakes of whole grains, fruits, and vegetables (especially dark green leafy vegetables) [9–11]. However, this advice is difficult for adults to comply with, let alone young children whose intake patterns are largely driven by taste preferences, not health considerations [12].

Two major factors conspire to predispose children to consume diets high in sugar, fat, and salt that may lead to obesity. First, humans have an evolutionarily drive toward heightened preferences for sweet and salty foods and rejection of bitter tastes. Second, children must be repeatedly exposed to the flavors of healthy foods beginning early in life to promote their acceptance of these foods.

Flavor Biology in Children

Biological Substrates of Flavor Learning

The perceptions arising from the senses of taste and smell combine in the oral cavity to determine flavor. These perceptions are often confused and misappropriated with olfactory sensations such as vanilla, fishy, and strawberry being erroneously attributed to the taste system per se when, in fact, much of the sensory input is due to retronasal olfaction. Because these senses are the major determinants of whether young children will accept a food (i.e. children eat only what they like), they take on even greater significance in understanding the bases for food choices in children than they do for adults.

We now know that flavor perception develops and functions in utero, and the senses of taste and smell continue to develop postnatally [13, 14]. The fetus begins to swallow and inhale large amounts of amniotic fluid around the 12th week of gestation [15, 16], and by the last trimester the receptors underlying taste and odor perception begin to communicate with the central nervous system in response to a variety of taste and odor stimuli [for a review, see 14]. Amniotic fluid, the first food of infants, contains a wide range of nutrients, such as glucose, fructose, lactic acid, fatty acids, and amino acids [17], as well as flavors (for which the odors are perceived retronasally) of the foods consumed by the mother [18, 19]. The fetus can detect these tastants and flavors, as infants prefer flavors previously experienced in amniotic fluid [18, 20–22].

Fetal swallowing frequency increases in response to the introduction of sweet solutions into the amniotic fluid and decreases in response to the introduction of bitter solutions [17, 23], which may be one of the first indications that our basic biology favors consumption of sweet tastes and avoidance of bitter tastes. A similar response pattern is seen shortly after birth. Within hours and days of being born, young infants react as would be expected to

pleasurable and aversive taste stimuli [24–33]: provision of sweet or umami solutions to neonates elicits rhythmic tongue protrusions, lip smacks, lip and finger sucking, and elevation of the corners of the mouth, all of which have been interpreted as a positive or hedonic response [27, 31]. In contrast, neonates gape, wrinkle their noses, shake their heads, flail their arms, and frown in response to a bitter solution [27, 29]. Concentrated sour solutions elicit lip pursing and, to a certain extent, gaping, nose wrinkling, and arm flailing as well as tongue protrusions and lip smacking [27, 29, 34]. Unlike the other basic tastes, neonates respond neutrally to salt taste – the taste for salt does not emerge until later in infancy and then remains throughout childhood and adolescence [35].

These specific affective reactions to differing taste stimuli are strikingly similar across cultures [25, 34, 36] and species [27, 37–40], also suggesting a basic biological underpinning for the flavors and foods youngsters prefer and avoid. Thus, when we examine children's dietary patterns from the perspective of the ontogeny of taste development, the foods children naturally prefer are not surprising and reflect their basic biology.

Heightened Sensitivity to Bitter and Preferences for Sweets and Salt in Young Children

Like infants, children live in different sensory worlds than do adults. Children have higher preferences for sweet [41–43], salt [44], and sour [45] tastes and are more rejecting of some bitter tastes [42] than adults. A vast amount of learning occurs during infancy and childhood, and a significant portion of that learning is about what and how to eat. Thus, reactions to taste qualities likely evolved to detect and reject that which is harmful and to seek out and ingest that which is beneficial [46]. Sweetness is associated with readily available calories from carbohydrate sources such as mother's milk or fruits [47], and saltiness is associated with needed minerals [48], whereas bitterness signals toxins and poisons [49]. Hence, from an evolutionary perspective, it makes sense that preferences for sweet and salty foods are inborn while preferences for bitter-tasting foods (e.g. coffee, dark green vegetables) are learned. It also makes sense that it would be protective for young children, who are trying to learn about what and how to eat, to be more sensitive to the cues proffered by foods; this heightened sensitivity would allow them to quickly protect them from that which causes harm and to encourage them to eat that which is beneficial for growth.

Only very recently in human history are foods omnipresent in many parts of the world and readily available for consumption. Rather, our taste preferences evolved in times of 'feast or famine'. Under such circumstances, preferences for sweet and salt and aversion to bitter were essential for ensuring that energy- and nutrient-dense foods were consumed and harmful substances were avoided. Now, in many parts of the world, a mismatch exists between children's physiology and the current food environment: many children live in

an environment that provides food everywhere – it is inexpensive, good tasting, and served in large portions. Further, the increased levels of sugar, fat, and salt in processed foods cater to children's natural taste predispositions.

Flavor Learning in Children

Sensory and biological considerations shed light on why children are predisposed *not* to favor low-sugar, low-sodium, and vegetable-rich diets and why it is difficult for children to eat nutritious foods when they are unfamiliar and do not taste good to them. However, while we cannot easily change children's basic biology, we can modulate children's flavor preferences by providing early exposure, starting in utero, to a wide variety of healthy flavors available within the culture.

Flavor Learning in Amniotic Fluid

Learning from mother is a fundamental feature of all mammals [50, 51]. In part, young mammals learn about things like body control, fine and gross motor movements, and social behaviors from what is modeled or transmitted by their parents [52]. Learning about flavors and foods is no different: young mammals first learn about what and how to eat through information transmitted by mothers, and these lessons come in many different forms.

Amniotic fluid is the first medium for flavor learning within which offspring experience the flavors of the mothers' diet. Flavors and chemicals consumed by the mother appear in the amniotic fluid [18, 19], and the fetus detects and responds to them. Human infants orient toward the odor of their own amniotic fluid within days of birth and prefer this odor to new odors experienced during the first few days of formula feeding [53, 54]. Shortly after birth, infants will respond differently to flavors experienced in amniotic fluid. For example, neonates whose mothers consumed an anise-flavored beverage or ate garlic-containing foods throughout pregnancy were more accepting of and interested in (as measured by mouthing and orienting) anise and garlic odors [21, 22]. Similar findings were observed with alcohol odors [20].

That these early flavor experiences can influence the acceptance of foods was first demonstrated in a randomized, controlled study of mothers who consumed carrot juice or water during their last trimester of pregnancy. Infants of mothers who consumed carrot juice were more accepting of carrot-flavored cereal at 5–6 months of age than were infants of mothers who consumed water [18]. In animal models, the influence of flavors experienced in utero has been shown to persist into adulthood, even without subsequent postnatal influence with the flavor [55, 56]. Thus, flavor learning begins in utero, long before actual experience with solid foods.

Flavor Learning with Mother's Milk

Flavor learning continues when infants experience the flavors of the mother's diet transmitted in breast milk. To date, many flavors (e.g., anise, garlic, ethanol, carrot, mint, vanilla, bleu cheese) have been empirically shown to pass from mother to offspring through the breast milk of many types of mammals [18, 50, 57–66]. Human infants detect the flavors in mother's milk, as evidenced by changes in their suckling rate, patterning and duration of feeding and intake [18, 62–64, 66], and differential acceptance of similarly flavored foods [18, 67].

At weaning, similar to other mammals [for review, see 50], human infants show greater liking for and acceptance of flavors and foods to which they have had early exposure. They were more accepting of cereal if it was prepared with mother's milk or if it contained a flavor (e.g. carrot) previously experienced in mother's milk [68, 69]. Similarly, breastfed infants were more accepting of fruits and vegetables than were formula-fed infants, but only if their mothers regularly ate these foods themselves, thus highlighting the importance of a varied diet for both pregnant and lactating women [67].

Several experimental studies have also shown that when breastfed or formula-fed infants are repeatedly exposed to a single fruit or vegetable for anywhere between 9 and 20 days, their preference for that fruit or vegetable increases and is higher than in infants who were not repeatedly exposed to that food [67, 70, 71]. Similar effects have been observed in studies with pre-school-age children [72–78].

Exposure to a variety of flavors, not just repeated exposure to a single flavor or food, also appears to facilitate acceptance of novel foods. Infants who were repeatedly exposed to a different starchy vegetable each day ate as many carrots after the exposure as did infants who were repeatedly exposed to carrots [70]. Similarly, repeated experience with a variety of fruits enhanced acceptance of a novel fruit but had no effect on infants' acceptance of green vegetables [71]. Because rejection of bitter taste is innate, infants may need actual experience with bitter taste, or more exposures, to enhance acceptance of green vegetables [67, 71]. That varied experiences with food flavors increase food acceptance may help explain why children who were breastfed are less picky during childhood [79].

Flavor Learning with Formula

Flavor learning is not specific to breastfed infants, as formula-fed infants learn to prefer the flavors of the formulae they are fed. However, formula does not have the variety of flavors experienced in breast milk. Further, the flavors of the different formulae may predispose infants to develop preferences for particular flavors. Traditional cow milk-based formulae (CMFs) have low levels of sweet, sour, and 'cereal-like' flavors; soy formulae have a combination of sweet, sour, and bitter flavors, and protein hydrolysate formulae (PHFs) have a combination of sour, savory, and bitter flavors and odors [80, 81]. PHFs are unpalatable to

older children and adults who have not had prior experience with them, but if introduced early enough, these formulae are preferred by infants who fed them [81]. That children attach to the flavor of the formula is evidenced by findings that they prefer the specific formula they were fed throughout infancy [82, 83].

Flavor Learning at Weaning

The type of formula an infant feeds modifies the infant's taste preferences both at weaning and later in life. PHF-fed infants showed greater acceptance of savory-, bitter-, and sour-tasting cereals during weaning [84]. Additionally, 38% of breastfed infants and 25% of infants who were fed CMF gaped while eating bitter-flavored cereal, while none of the infants fed PHF made this facial expression of distaste [84]. The effects of early exposure to such flavors were particularly persistent, leading to heightened preferences for the taste and aroma of the formula as well as foods that contain similar volatiles or tastes (e.g. broccoli, chicken) several years after children's last exposure [83].

Children at weaning look to their mothers to learn about what and how to eat. Research in animal models reveals this learning can be quite complex. For example, calves or lambs that see their mother avoiding larkspur also do not eat this plant [85]. Mothers serve as models to their young, teaching them which plants to avoid and when plants are at their peak nutritional content [86]. In humans, the extent to which mothers consume healthy foods and make these foods available to their children is positively correlated with their children's intake of healthful foods [79, 87–89]. Experimental studies have provided strong support for the influence of adult models on young children's acceptance of novel foods [90–93], further demonstrating that children are primed to learn the flavors and foods made available by mothers.

Sensitive Periods in Human Flavor Learning

Based on the evidence cited above, it is likely that early human flavor exposure, particularly in utero and in the context of breastfeeding, influences later flavor acceptability. Although there is emerging experimental evidence supporting this hypothesis [18, 67], recent studies, which exploit the inherent flavor variation in infant formulae, have revealed an apparent 'sensitive window' during the early infancy for increased acceptance of a complex flavor – that of PHF [81].

PHF, developed a half-century ago, is currently the feeding regimen of choice for formula-fed infants who cannot tolerate cow milk and other intact proteins [94]. Pediatricians have remarked anecdotally that although it is easy to introduce this type of formula to infants during the first weeks of life, it becomes extremely difficult to do so later in infancy [95]. This early acceptance has been attributed to the young infant's 'lack of taste perception' since these formulae are reported by adults to have an extremely unpalatable,

offensive off-flavor. However, as discussed above, basic research in taste has shown that young infants will reject extreme sour [29] and bitter tastes [96] and they can detect a variety of odors such that their sensitivity may equal or surpass that of adults [for a review, see 14].

During the past two decades, we have experimentally investigated the age-related changes in the infants' willingness to feed PHF [81, 97, 98]. By comparing the acceptability of PHF by infants at 2 months and then at 7 months of age, we found that the younger infants were clearly willing to accept substantial amounts of PHF and fed to satiation, as observed previously [99, 100], even though they could detect the difference between the PHF and their familiar CMF. However, these infants invariably rejected PHF when retested at 7 months of age. This rejection was evident within the first minute of the feed, suggesting that the sensory qualities of the formula were responsible, at least in part, for the rejection.

To better characterize the timing of the sensitive period, we conducted a randomized clinical trial that varied the age and duration of PHF feeding [81]. This revealed a 'window' of acceptance during early life when infants readily accept PHF. Then, beginning around 4 months of age and continuing through adulthood, its flavor is rejected unless the individual has been exposed to PHF during early life. That is, PHFs have a completely different hedonic tone depending on whether the infant was exposed to this formula during the first few months of life.

Why should there be a sensitive period in the early acceptance of hydrolyzed formulae? First, presuming there is an adaptive reason, it clearly has nothing directly to do with hydrolyzed protein formulae, which were introduced only a half-century ago. Indeed, these observations with formulae may conveniently expose a much more fundamental aspect of early mammalian flavor learning. We hypothesize that it is important for the human infant to accept and be particularly (but not exclusively) attracted to the flavors that are consumed by the culture and, more specifically, by the mother. All else being equal, these are the flavors that are associated with nutritious foods or, at the very least, foods the mother has access to – and the foods and flavors that the infant will experience at weaning and probably thereafter. Under this hypothesis, much of the normal exposure would occur in utero and during breastfeeding, where flavors mothers consume are transferred to these chemosensory environments. Additional research is needed to determine the extent to which early exposure to these flavors, perhaps during sensitive periods of development, helps establish enduring preferences for foods and flavors.

Conclusion

The unhealthy eating habits that plague adults also contribute to the increasing global prevalence of obesity among children. Although children

have an evolutionarily driven heightened preference for sweet and salty foods and for rejection of bitter tastes, research shows that repeated, early exposure to the flavors of healthy foods may help promote their acceptance of these foods later in life. Taste and flavor perception develop and function in utero, and flavor learning continues when infants experience the flavors of the mother's diet in breast milk and the flavors of formulae. Children just beginning to consume solid foods look to their mothers to learn about what and how to eat. Based on the evidence cited in this chapter, it is likely that flavor exposure before 4 months of age, including repeated exposures to a wide variety of healthy foods, and good eating behaviors modeled by parents will modify the infant's food preferences both in childhood and later in life. Thus, early life represents a critical window of opportunity to influence healthy food choices throughout an individual's lifetime.

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Discussion

Dr. Haschke: One comment related to terminology. You consider Nutramigen to be a PHF, i.e. a partially hydrolyzed formula.

Dr. Mennella: PHF is often used to symbolize protein hydrolysate formula.

Dr. Haschke: Nutramigen is an extensively hydrolyzed formula (EHF).

Dr. Mennella: PHF is used to denote protein hydrolysate formula which can be further specified as pPHF = partial hydrolysate formulas, and ePHF = extensively protein hydrolysate formulas.

Dr. Haschke: Another comment is on growth. We have good data from the GINI study in Germany where 3,000 infants were followed until 10 years of age. They were either breastfed, received partially hydrolyzed formula, extensively hydrolyzed formula, or cow's milk-based formula. There was a transient effect on growth. It disappeared completely at 5 years of age. Children no matter what the initial feeding was had the same body mass index, same height. Now the data are out for 10 years, and again there is no difference between the groups.

Dr. Mennella: Many infants in the past studies were also breastfed confounding the findings. The most recent study was a randomized clinical trial that consisted of term infants who were exclusively formula fed.

Dr. Haschke: But I am just referring to the GINI study.

Dr. Mennella: When we looked at the longer term growth, these effects seem to go away by 5–6 years. I think that the differential growth patterns (specifically the more accelerated growth among infants fed cow's milk formulas) are still quite important. I would focus on what these data are telling us about infant satiety and satiation. There is a convergence of findings from these trials showing difference in growth patterns based on the type of formula fed during the time in life when formulas are these infants' primary source of nutrition. For the first time, we found that differences in growth may be due in part to differences in satiation when feeding ePHF. To me, that's a fascinating discovery.

Dr. Saavedra: I have two comments. One is that when studying these things we need to be absolutely clear about what it is that we are testing. The other is the percentage of amino acids in these formulas. Most extensive hydrolysates, for example, have between 40 and 50% free amino acid as opposed to partial hydrolysates which have hardly any. Intact proteins, which actually do have some free amino acids, probably also have some taste influence. So here again, the generalizations are going to be tough when it comes to those.

Dr. Mennella: We analyzed a number of formulas, and each formula has its own different free amino acid profile which may contribute to the different taste profiles, so I agree.

Dr. Saavedra: So again, this is just for the purposes of specificity when we are reporting these kinds of results. But the other question that you bring up is more fascinating. What, if anything, can we do for a child that is getting the same thing, whatever it is that they are getting when they are not breastfeeding? Are there for example data or experience on variety of acceptability of tasting from children who are breastfed versus children who have been on a formula for a period of time?

Dr. Mennella: Dr. Birch has done work showing that breastfed infants were more accepting of a new food and were less picky eaters as they grew. We have conducted a number of experiments on infants at the time of weaning. Infants learn through repeated exposure, through experiences with dietary variety and through flavor experience in breast milk. Breastfeeding is clearly giving the baby an advantage for food acceptance. If the mother eats fruits and vegetables, their baby is more accepting of those foods. The mother has to eat the food in order to have an effect on the infant.

It's a beautiful and elegant system which should be modeled throughout all of the lifespan.

Dr. Kleinman: Salty taste seems to be much better tolerated in infancy than later on. Oral rehydration solution, for example, is well accepted by infants but pretty hard to give to anybody over the age of 1. Thus, there appear to be thresholds over time where excessively sweet and salty tastes are no longer accepted. Do we know the biological basis for that?

Dr. Mennella: While babies are born preferring sweet taste, they cannot detect salt tastes until they are 4 months of age. From that time onwards, children prefer salt and sweet taste. Now, when it comes to the intensity of sweetness or salt that a child likes, a child will prefer a much more intense sensation. Children prefer, on average, a 0.6 M sucrose solution; for reference, the level of sweetness in a Coca Cola is 0.3 M. The same is true for salt – they prefer much higher levels than adults. Children are clearly living in different sensory worlds than adults!

Dr. Kleinman: And that's a learned behavior?

Dr. Mennella: No, I think it's their basic biology. I think that the child is attracted to sweet and salt during periods of growth; sweet is our taste signal for carbohydrates and salt is our taste signal for needed minerals.

Dr. van Goudoever: With regard to hydrolyzed formula and complementary feeding tolerance, from what I understood you have to try offering it over and over again, and basically the turning point is when you've offered it about 8 times; then there is some acceptance. What are the newest data on that, and does that account for hydrolyzed formula as well?

Dr. Mennella: It's extremely difficult to introduce ePHF when the baby is 4 months of age or older. It's not that it can't be done but it's difficult. Common strategies (that are not evidence based) include mixing ePHF with cow's milk formula and then gradually increasing the amount of ePHF in the mix over time. Now, when it comes to complementary foods, and I think you will hear more of this tomorrow, 8–9 exposures result in greater acceptance. Merely looking at the food is not sufficient. Rather, children had to taste the food to learn to like it. We do not know the minimum amount of exposure needed and I would hypothesize that, depending on the food, it may be different for a child based on his/her taste sensitivity. For example, children who are more sensitive to bitter tastes may need more exposures.

Dr. van Goudoever: Then a further short question, what kind of growth charts did you use in the pediatrics tables?

Dr. Mennella: The WHO and then we did CDC too, the results aren't changed.

Dr. Stettler: I like your comment on the two things that are going on right now in the US. One of them is new formulas that have been marketed for children as young as 10 months that are flavored with chocolate and vanilla. It looks like they are now marketing it to 12 months or even 24 months and after. And then the other thing is the heavy marketing for school age children by the National Dairy Council of chocolate-flavored sweetened milk in the schools. So I would like to hear your comments on that. Obviously, these are periods that are further down than what you studied, so should we not be concerned at all about long-term choices after a certain age, and what would that age be?

Dr. Mennella: I don't know what that magic age is, but I think that learning about foods begins very early. We first learn about flavors of our mothers' diet during pregnancy and lactation. These are the foods that a part of the culture in which the child is born and will be the foods that are offered to the child as he or she grows. For children, acceptance of many foods/products is enhanced if you make it taste sweeter. Through experiences with these sweetened products, children are learning that these milks should taste sweet. I often wonder what the consequences of feeding these flavored

follow-on formulas have on children's acceptance of cow's milk. There is inherent plasticity in the senses which tell us this food tastes good, this food doesn't, and these experiences are particularly salient during their early development. In all cultures around the world, children's diets are usually a modification of the adult diet. It has only been in recent history that we are starting to feed children different types of food. I think we have to take a lifespan approach to feeding and realize that these foods are teaching the child the food culture of their families.

Dr. Lack: I am intrigued by the evolutionary significance of this period of gustatory promiscuity from 0 to 4 months. What are the implications of this? Is it that infants less than 4 months should be allowed to touch, smell and perhaps even taste complementary foods, or is it all mediated through the mother's diversity in her diet during pregnancy and lactation? Is there an accompanying drive in the mother's appetite? Does her behavior change in pregnancy and does she start eating more fruits, could it explain cravings for certain foods?

Dr. Mennella: These are excellent questions and there is not a lot of experimental work. I think that if we were looking at other animals, and especially some of the work that is coming out of wildlife and field biology, the first way animals learn about flavors is in amniotic fluid and the mother's milk. But it's the first way, not the only way. Learning about foods continues, and if these foods continue to be experienced in a variety of contexts, this learning gets reinforced as the child grows. So we can't just expect to give the experience with the flavor of broccoli in formula, for example, and expect that their preference for broccoli is going to remain strong even if the mother doesn't eat broccoli or provide broccoli for the child to eat. It's a gradual process that builds on the familiar; it's the most fundamental aspect of learning.

Dr. Zlotkin: Is there a relationship between taste and hunger and appetite? In my clinical practice, I see children with a very maladapted behavior, that is children who seem not to have any appetite and actually refuse to eat. Often, they are survivors of preterm birth, but sometimes they are not and they seem to come from families whose background is seemingly normal, and I continue to be surprised by these children who seem absolutely unwilling to eat, have no appetite, and I am just wondering if there is a relationship between taste, hunger and appetite.

Dr. Mennella: There again, I looked at the non-human animal studies when I first designed this work on human infants. I always tested the babies a half hour to an hour before they are scheduled to feed because if you test them when they are very hungry taste isn't a salient. Another important issue is whether there are sensitive periods for learning about foods. In particular, what happens if you are deprived of these experiences, as in the case of tube-fed infants who often have difficulty with later feeding. This is an important area that needs research.

Dr. Chittal: I was just wondering, it's a little simplification of the very complex issue of taste, but taste is an acquired parameter almost like the heart function, and babies who are on hydrolyzed protein formula find it easy to accept a similar formula later on in life. Having been used to the taste of hydrolyzed protein formula do they find it difficult to accept cow's milk formula later on? I will ask a second question if you don't mind. In the previous papers on the early introduction of raw cow's milk, I suspected that this introduction was by default or by choice because many of the infants who have been on cow's milk formula refuse to accept it after a particular amount of time, and they instinctively accept raw cow's milk. So, is there a taste factor?

Dr. Mennella: I will speak to the protein hydrolysate question first. We have followed these children for several years; these children are more accepting of ePHF flavors and sour and bitter tastes several years after their last ePHF feeding. Also, there is a volatile in ePHF that is found in broccoli, and we found that children who

are fed protein hydrolysate tend to like broccoli more. Other reports of long-term effects of feeding PHF come from the literature on children and teenagers who have phenylketonuria; nutritionists report that they prefer sour flavors added to the formula diet. I think that through these flavor experiences in milk or formula, children develop preference for flavors they experience. It raises a very interesting question: How different does the formula have to taste for the infant to detect a difference in it? Unlike formula which is rather monotonous from day to day, the flavor of breast milk changes throughout the course of a feed (the fat content more than doubles) and changes from meal to meal since the mother's diet is changing from morning to evening, etc.

Dr. Shreffler: In patients that I see who have multiple food allergies and food aversions, I have thought of that as largely a motor issue, and have made the point to intervene with chewing, etc. Obviously, your data suggest that taste is to some extent part of that development of aversions. What is known about interactions between receptors for histamine, substance P, other mediators, and taste receptors, because it's a striking clinical observation that even after outgrowing food allergies these kids often have very strong aversions to specifically those offending foods.

Dr. Mennella: I don't know of anything. But I think it's extremely important because if there is learning that's occurring, one would imagine that these sensory features could trigger other types of responses. Unfortunately, I don't know of any studies that have looked at that.

Dr. Guandalini: I just wanted to add a comment on the early onset of preferences toward sugary taste. In reality, if one looks at the ontogeny of the human intestinal disaccharidases, one is confronted by the fact that the enzyme sucrase isomaltase (that digests sucrose, a carbohydrate only found in the plant kingdom) is developing very early on so that at term the activity of sucrase is comparable to that of adults [1]; it looks like mother nature makes us geared towards early utilization of sugars.

Dr. Mennella: Whether you look at the near-term fetus, the premature infants, newborns, the convergence of findings from countries around the world is that babies like sweet and it's part of their biology. That is, the liking for sweets is not a consequence of modern day food environment. I think what the food industry has done is provided more experiences for children to learn the context for sweets. However, they did not create the inborn liking of sweets.

Dr. Klish: In my early days of treating children with feeding aversions, our ability to concentrate calories was limited. Infants with chronic congestive heart failure from congenital heart disease were very difficult to feed, and their growth mandated their eligibility for corrective surgery. The only additives I had to increase the caloric content of formula were vegetable oil and maltodextrin. Many of these children developed an aversion to sweetness because everything they ate had maltodextrin added. So the ability to tolerate sweet obviously has a limit.

Dr. Mennella: Did they get sick?

Dr. Klish: Yes, they would get sick in the sense that they would regurgitate the formula as well as refuse it. They looked like the child you had on your slide.

Dr. Mennella: Yes, that's taste aversion learning; how the consequence of the taste experience (illness) can shift its hedonic tone; it's a powerful mechanism.

Dr. Stathatos: Are early gustatory signals in any way connected with the later high carbohydrate consumption, French fries and so on?

Dr. Mennella: Children like more intense sweetness and industry seems to be catering to their basic biology. Since the 19th century, the first thing a child has ever bought with their money is a candy. I think it also speaks to the saliency of this taste in a life of a child. It's the sweet taste of childhood.

Reference

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Early Feeding Practices and Development of Food Allergies

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Abstract

Despite increasing efforts to prevent food allergies in children, IgE-mediated food allergies continue to rise in westernized countries. Previous preventive strategies such as prolonged exclusive breastfeeding and delayed weaning onto solid foods have more recently been called into question. The present review discusses possible risk factors and theories for the development of food allergy. An alternative hypothesis is proposed, suggesting that early cutaneous exposure to food protein through a disrupted skin barrier leads to allergic sensitization and that early oral exposure of food allergen induces tolerance. Novel interventional strategies to prevent the development of food allergies are also discussed.

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Introduction

Recent epidemiological studies in the UK and North America have shown that prevalence rates of food allergy in children have increased. Food allergy prevention through allergen avoidance during pregnancy, breastfeeding, and infancy has been seen as an effective public health policy to prevent allergies. Nonetheless, there are little epidemiological data to support this recommendation. Interventional trials on dietary elimination have failed to reduce IgE-mediated food allergies. Conversely, there are preclinical data and some clinical data to suggest that early oral exposure results in the induction of tolerance. New strategies to prevent food allergy in infants need to be put to test in randomized controlled interventional studies. In this review, we analyze potential risk factors and theories for the development of food allergy.

Rise in Food Allergies

Since the late 1950s, the incidence of allergy in developed countries has risen progressively. In the US, the prevalence of reported food allergy increased by 18% from 1997 through 2007 in children less than 18 years of age ($p < 0.01$) [1]. Ambulatory care visits due to allergy tripled between 1993 and 2006 ($p < 0.01$). In 2007, 3.9% of US children <18 years of age had reported food allergy [1]. In the UK, food hypersensitivity prevalence based on food challenges and appropriate clinical history is 5–6% by the age of 3 years [2]. Trends in hospital admissions in the UK show that admissions for food allergy in children rose nearly 7-fold from 16 to 107 per million for the time period 1990–1991 to 2003–2004 [3]. The increase in peanut allergy (PA) has been significant. In the US, the rate of self-reported PA increased from 0.6 to 1.4% among children from 1997 to 2008 [4, 5]. In the UK, a recent study including 1,072 mothers and their children showed that the prevalence of peanut sensitization is 2.8% and the prevalence of PA is 1.8% among British children at school entry [6].

Epidemiologic Risks for Food Allergy

There are diverse theories that try to explain the presence and rise in allergies during the past few decades.

Genetic and Molecular Risk Factors

Some studies suggest a strong genetic contribution to PA. A child has a 7-fold increase in the risk of PA if he or she has a parent or sibling with this allergy [7]. In the case of monozygotic twins, a child has a 64% likelihood of PA if his or her twin sibling has PA [8]. Although it is unlikely that genetic risk factors could account for the recent increase in food allergies, it is nevertheless likely that there are genetic predisposing factors for their development. The contribution of the HLA background and the development of individual food allergies to the rise of food allergies remains to be seen.

Changes in Dietary Composition

In the past 3 decades, there have been marked changes in diet, and it has been suggested by researchers that differences in macronutrient and micronutrient dietary content could explain the increase in allergies. There are 3 hypotheses that deserve discussion:

- 1 The vitamin D hypothesis is based on both epidemiological and immunologic data that suggest that either excessive vitamin D or conversely vitamin D deficiency has led to increased allergies. The first observations derived from farming communities in Germany where there was less vitamin D supplementation used in foods and a lower prevalence

of allergies in children was found. Allergies increased coinciding with vitamin D supplementation intervention programs to prevent rickets in childhood [9]. Similarly, two independent cohort studies by Milner et al. [10] and Hyppönen et al. [11] showed that infants who had vitamin D supplementation were at increased risk of food allergy. On the other hand, the vitamin D deficiency hypothesis argues that inadequate vitamin D (mainly as a result of inadequate sunlight associated with more time indoors) is responsible for the increase in asthma and allergies. The study by Camargo et al. [12] found a strong north-south gradient for EpiPen (Dey, Napa, Calif., USA) prescriptions in the US. Northernmost states were prescribing 8–12 EpiPen self-injectors per 1,000 population, whereas the southern states were prescribing 3 per 1,000 population. This gradient persisted despite a multivariate analysis. There was an inverse association between EpiPen prescription and the incidence of melanoma in the population, suggesting that this north-south effect was due to sunlight exposure [12].

- 2 The dietary fat hypothesis argues that reduction in consumption of animal fats and corresponding increase in the use of margarine and vegetable oils has led to the increase in allergies. The argument is that there has been an increase in the consumption of ω -6 polyunsaturated fatty acids, such as linoleic acid, and similarly that through reduced consumption of oily fish, there has been a reduction in ω -3 polyunsaturated fatty acids, such as eicosapentaenoic acid. ω -6 fatty acids lead to the production of prostaglandin E_2 (PGE₂), whereas ω -3 fatty acids inhibit synthesis of PGE₂. PGE₂ reduces IFN- γ production by T lymphocytes, thus resulting in increased IgE production by B lymphocytes [13, 14].
- 3 The antioxidant hypothesis proposes that the decrease in consumption of fresh fruit and vegetables (containing antioxidants such as vitamin C, vitamin E, β -carotene, selenium and zinc) in the UK might account for allergies. However, dietary trends are conflicting; whilst the intake of some antioxidants has increased, for others intakes have decreased. Nevertheless, there is epidemiological, animal, molecular and immunological evidence suggesting associations between antioxidants and asthma and a reduced number of studies on atopic dermatitis and allergic rhinitis [15]. However, no such data are currently available for food allergies.

Hygiene Hypothesis

In general, allergies are associated with a western style of life. The hygiene hypothesis suggests that the lack of early childhood exposure to infectious agents, gut flora and parasites increases susceptibility to allergic diseases by modulating immune system development. Limited evidence for the hygiene hypothesis exists with respect to food allergy. A Norwegian birth cohort study found that birth through a cesarean section was associated with a 7-fold increased risk of parental perceived reactions to eggs, fish, or nuts [16]. A

recent meta-analysis found 6 studies that showed a mild effect of cesarean delivery, increasing the risk of food allergy or food atopy (OR: 1.32, 95% CI: 1.12–0.55) [17]. One explanation for these findings is that early colonization of the infant by colonic microflora protects against the development of allergic disease. Such observations have led to strategies to alter commensal gut flora either directly through the administration of probiotics or indirectly through the administration of prebiotics. Although some studies using probiotics have suggested some protective effect against development of eczema, they did not show any reduction in allergen sensitization [18]. There is no evidence that probiotics prevent the development of food allergies. However, cesarean section could appear to cause a higher rate of food allergy and atopy due to confounding factors that have not been analyzed in the published literature. Firstly, cesarean sections are associated with higher maternal age which in itself has been linked to atopy [19]. Secondly, cesarean section is associated with a higher number of first born infants which has been shown to be associated with food allergy [20]. Finally, cesarean sections are associated with a higher number of male births [21] and the prevalence of FA is higher in males than in females [22].

Exposure to Food Allergens

Important questions remain about exposure to food allergens both in the infant's diet and in the maternal diet. The American Academy of Pediatrics recommended until very recently that families with an infant at increased risk of atopy based on family history should avoid peanuts in the infant's diet during the first 3 years of life and common food allergens until the first (milk), 2nd (egg), or 3rd (tree nuts and fish) years of life [23]. According to these recommendations, mothers should avoid peanuts during pregnancy and breastfeeding and additional allergens during lactation. In the UK, similar recommendations are still in place with respect to peanut avoidance [24]. Recently, both the American Academy of Pediatrics and the section on Paediatrics of the European Academy of Allergology and Clinical Immunology have changed their position, acknowledging that we do not know whether certain aspects of avoidance prevent allergies, and recommendations about avoidance of specific food allergens have been withdrawn and replaced by comments about the lack of current evidence on these topics [25, 26].

There is a lack of evidence in which to base advice for weaning infants. We have little evidence-based guidance about when to introduce allergens in the diet and whether to introduce these foods in large or small quantities, and what the frequency should be. The World Health Organization strategy to prevent allergy is to promote exclusive breastfeeding during the first 6 months of the infant's life and thus delay weaning onto solids and milk for-

mulae [27]. However, there is no convincing evidence that exclusive breastfeeding beyond 4 months of age has any effect on reducing atopic disease. Indeed, more recently, observational cohort studies showed that breastfeeding [28] and prolonged breastfeeding [29] are associated with an increased risk of asthma and eczema. Although such studies do not eliminate the possibility of reverse causality as an explanation for this finding (high-risk infants with eczema are deliberately breastfed longer), they raise the question as to whether exposure to solids in infancy might have a role in preventing allergic disease.

Food Allergen Exposure Revisited

It is surprising that studies eliminating food allergens during pregnancy, lactation, and infancy have consistently failed to reduce long-term IgE-mediated food allergy in children [30]. There are four possible explanations for this failure. First, exposure to allergens is irrelevant for the development of food allergy. This explanation can be immediately ruled out because food allergy is an antigen-specific immunologic disease, and antigen exposure is necessary for T cell maturation, affinity maturation, and isotype switching. Second, allergen reduction measures have not been sufficient in previous studies, and dietary elimination was not sufficiently stringent. This is certainly plausible. However, it seems very unlikely that ‘complete’ allergen avoidance could successfully prevent food allergies as a public health measure, given that careful elimination studies have failed, despite rigorous dietary supervision, to achieve a reduction in food allergies [30, 31]. Third, sensitization to food allergens does not occur as a result of consumption, but can occur through other routes of exposure. This is supported by a number of murine studies that show that allergic sensitization to antigen occurs after cutaneous exposure and also this has been suggested in recent clinical studies. Finally, the paradigm of allergen avoidance is flawed, there are animal data and some observational clinical data supporting that early oral exposure can be required to prevent the development of allergy. These last two explanations are discussed further below.

Dual Allergen Exposure Hypothesis

The established view that allergic sensitization to food occurs through oral exposure and prevention of food allergies is best accomplished through elimination diets has been challenged. It is proposed instead that allergic sensitization to food can occur through low-dose cutaneous sensitization and that early consumption of food protein induces oral tolerance [32]. The timing and balance of cutaneous and oral exposure determine whether a child will have allergy or tolerance (fig. 1).

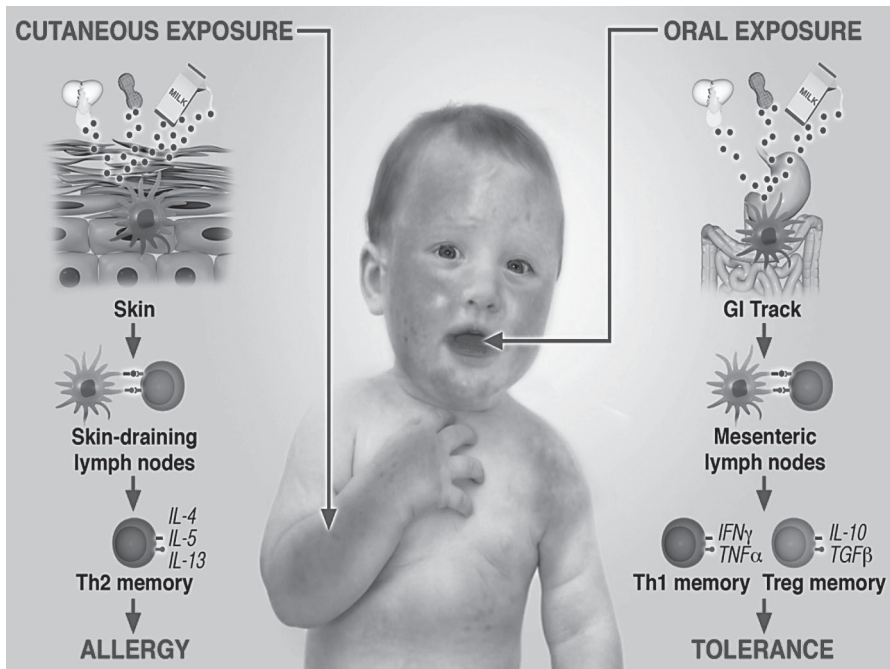


Fig. 1. Dual allergen exposure hypothesis for pathogenesis of food allergy. Allergic sensitization results from cutaneous exposure, and tolerance occurs as a result of oral exposure to food. Reprinted with permission from Lack [32].

Data Suggesting Cutaneous Sensitization

Current knowledge suggests that atopic dermatitis is the result of a combination of an altered skin barrier function, abnormal immune reactivity and environmental factors such as allergens and microbes [33]. There is indeed a molecular basis for the increased skin permeability in eczema; this is the loss of function or missense mutations in the gene encoding for filaggrin. This protein is important for epidermal differentiation, desquamation and barrier function and has been recognized as the strongest genetic contributor to eczema [33–36]. In the positive studies, it has been shown that 14–56% of cases of eczema carry one or more filaggrin null mutations, and the presence of a filaggrin null allele represents a 1.2- to 13-fold increased risk of developing atopic eczema [37]. Furthermore, there is evidence that TH2 inflammation in the skin of patients with eczema reduces filaggrin gene expression [38]. It has been suggested that low-dose exposure to environmental foods (on tabletops, hands, and dust) [39], penetrates the disrupted skin barrier and is taken up by Langerhan's cells. This leads to TH2 responses and IgE production by B cells [33]. This hypothesis can explain the association between the presence of early severe eczema in infancy and the subsequent development of food

Table 1. Food allergies among allergy clinic patients

Country	PA, %	Dietary practice recommendations (infant peanut consumption)
UK (n = 191)	25	avoidance
US (n = 300)	69	avoidance
Israel (n = 992)	2.1	high infant consumption
Philippines (n = 184)	0	high infant consumption
From Lack [32], with permission.		

allergies. Furthermore, this hypothesis can explain different rates of food allergies in different parts of the world and changes in food allergy over time. Thus, in societies in which a food is not consumed, there is no environmental exposure, and therefore allergy to that food will not occur. Allergy to kiwi was not a problem in the UK before it was introduced into the market in the 1970s through 1980s. In countries where consumption of peanut is high and peanut is therefore present in the environment but infants are avoiding peanuts, one would expect to see allergic sensitization (UK, US, Canada and Australia). In countries where consumption and consequently environmental exposure are high but infants are eating peanut regularly, one would not expect to see PA (southern/western Africa/Asia) [32] (table 1).

In animal models, it has been shown that exposure of mice to ovalbumin or peanut on abraded skin led to significant specific IgE responses [40, 41]. There are human studies in which food allergen-specific T cells have been isolated from lesional skin in patients with eczema [42]. In a prospective birth cohort study, it was found that low-dose exposure to peanut in the form of arachis oil applied to inflamed skin on infants was associated with increased risk of PA at age 5 years [43]. Similarly, transcutaneous sensitization to oat used in emollients or moisturizers was suggested in a study assessing children with atopic dermatitis. Thirty-two percent of children using creams containing oat had oat-positive patch test compared with 0% in the children who did not use them [44]. In a recent cross-sectional study [45], the relevant route of peanut exposure in the development of allergy was evaluated. Maternal peanut consumption during pregnancy, breastfeeding, and the first year of life was recorded by using a questionnaire; additionally, peanut consumption among all household members was quantified. The median weekly household peanut consumption in the peanut allergic cases was significantly elevated (18.8 g, n = 133) compared with controls without allergy (6.9 g, n = 150) and high-risk controls (1.9 g, n = 160; $p < 0.0001$). A dose-response relationship was observed between environmental (non-oral) peanut exposure and the development of PA. These findings suggest that high levels of environmental exposure to peanut during infancy may promote sensitization, whereas low levels appear to be protective

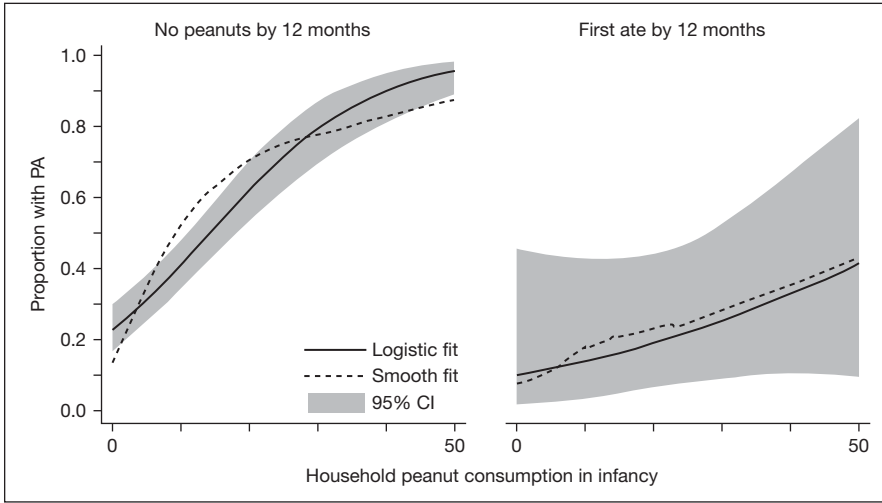


Fig. 2. PA among children with food allergy ($n = 293$) as a function of environmental exposure depending on whether child first ate peanuts by 12 months. Reprinted with permission from Fox et al. [45].

in atopic children. Early oral exposure to peanut in infants with high environmental peanut exposure may have had a protective effect against the development of PA. No effect of maternal peanut consumption during pregnancy or lactation is observed, supporting the hypothesis that peanut sensitization occurs as a result of environmental exposure (fig. 2).

Data Suggesting Oral Tolerance

Oral tolerance is well recognized in murine models. Numerous studies have demonstrated that early high-dose oral exposure confers both immunologic and clinical tolerance to food allergens. A single oral dose of allergen (β -lactoglobulin, ovalbumin, or peanut) is sufficient to achieve tolerance and prevent subsequent allergic sensitization [46–48]. In a murine model, a single high dose of peanut flour (100 mg) was sufficient to promote oral tolerance and prevent subsequent IgE sensitization and T cell proliferation [48]. In human subjects, cutaneous exposure to nickel during childhood leads to sensitization and nickel allergy, but oral exposure to nickel through orthodontic braces before ear piercing protects against nickel allergy [49, 50]. Similarly, subjects exposed to pancreatic extract by means of inhalation or contact have IgE-mediated allergic reactions, whereas subjects exposed orally do not [51].

Regular fish consumption before the age of one year appeared to be associated with a reduced risk of allergic disease [OR: 0.76, 95% CI: 0.61–0.94] and sensitization to food and inhalant allergens [OR: 0.76, 95% CI: 0.58–1.0] during

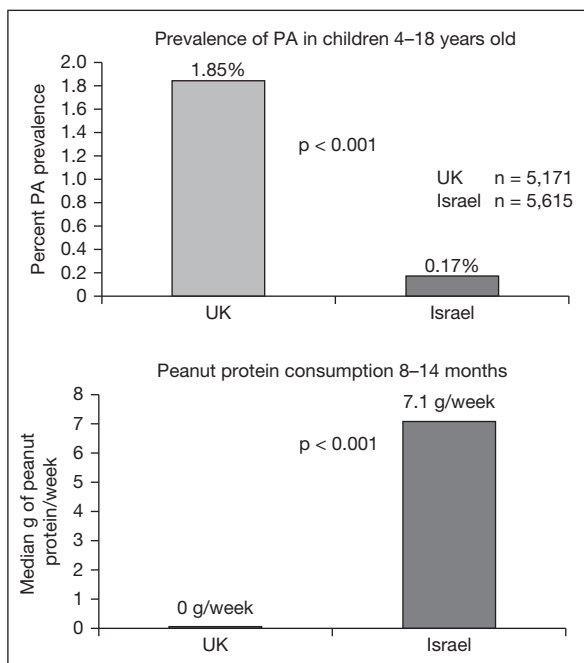


Fig. 3. Early consumption of peanuts in infancy is associated with a low prevalence of PA. Adapted from Du Toit et al. [60].

the first 4 years of life in a cohort of 4,089 newborn infants [52]. On the other hand, a recent study showed that delaying initial exposure to cereal grain after 6 months of life was associated with an increased risk of IgE-mediated food allergy [53].

In Western industrialized societies where peanuts are avoided in pregnancy and infancy, the rate of PA is higher [54]. In regions where peanut is consumed in high amounts during infancy (Middle East, Southeast Asia, and Africa), PA is reportedly rare [55–57]. However, different rates of food allergies in the UK compared with those in Asia and Africa might be due to genetic differences or the generally lower rates of atopic disease in developing countries, possibly resulting from differences in microbial exposure [58, 59]. In a recent cross-sectional study among Israeli ($n = 5,615$) and UK ($n = 5,171$) Jewish children [60], the prevalence of PA was 10-fold higher in the UK (1.85%) than in Israel (0.17%; $p < 0.001$; fig. 3). This study also found that peanut is introduced earlier and is eaten more frequently and in larger quantities in Israel than in the UK. The median monthly consumption of peanut in Israeli infants aged 8–14 months is 7.1 g of peanut protein, and it is 0 g in the UK ($p < 0.001$). The median number of times peanut is eaten per month was 8 in Israel and 0 in the

UK ($p < 0.0001$). This difference is not accounted for by differences in atopy, social class, genetic background, or peanut allergenicity. These findings raise the question of whether early introduction of peanut during infancy, rather than avoidance, will prevent the development of PA.

Randomized Controlled Trials Using Oral Tolerance Induction to Prevent Food Allergies

Indeed, it has been argued that early introduction of foods such as peanut may lead to tolerance and protect against the development of food allergy. These theories are currently being tested in two randomized controlled trials (RCTs).

The LEAP Study [61] (www.leapstudy.co.uk) involves 640 such high-risk children who were enrolled in the study when aged 4–10 months. Each child was randomly assigned to follow one of the two approaches – avoidance or consumption. Children in the avoidance group avoid eating peanut-containing foods until they reach the age of 3. In the consumption group, parents are asked to feed their child an age-appropriate peanut snack three times per week (equivalent to about 6 g of peanut protein per week). All participants receive allergy testing, dietary counseling, physical examinations and will be asked to provide occasional blood samples that will be used to examine differences in immune system development in each of the study groups. The proportion of each group that develops PA by 5 years of age will be used to determine which approach – avoidance or consumption – works best for preventing PA. We anticipate that the study will reach completion in 2013, at which time the results will be analyzed and published.

The EAT study [62] (www.eatstudy.co.uk) is an RCT investigating the effect of early introduction of complimentary foods together with breastfeeding. Infants taking part in the study ($n = 1,400$) are being recruited from the general population and randomized to one of two groups: one group ($n = 700$) introduces six allergenic foods from 3 months of age alongside continued breastfeeding, having been screened to check for pre-existing food allergy (early introduction group). The other group ($n = 700$) follows present UK government weaning advice, i.e. aim for exclusive breastfeeding for 6 months (standard weaning group). The children will be monitored until 3 years of age to see whether early diet has an effect on reducing the prevalence of food allergy determined by double-blind, placebo-controlled food challenges.

Pitfalls in the Interpretation of Randomized Controlled Trials to Prevent Food Allergy (Necessary and Sufficient Causes)

Interventional studies clearly represent an advantage over observational studies in the determination of the role of early food and micronutrient

exposure in the development of allergies. RCTs represent the gold standard of clinical medicine, especially when findings are replicated and shown to be consistent in further meta-analyses. However, we should bear in mind that positive RCTs are easier to interpret than negative studies. The pathogenesis of food allergies is likely to be multifactorial, and it is also likely that the induction of oral tolerance is dependent on several conditions being met. Thus, we need to differentiate between necessary and sufficient causality. Exposure to food proteins in the GI tract may require an optimal microenvironment, if the necessary conditions for the induction of tolerance are to be met (e.g. immune factors such as cytokines, antibodies, regulatory T cells whose function may depend on vitamin D, as well as bacterial colonization). For example, in animal models, oral tolerance induction with a single dose of food protein protects against the development of allergies. However, oral tolerance cannot be induced in germ-free mice – tolerance requires the presence of both intestinal microflora and food antigen [63]. Each factor is necessary but neither is sufficient for the development of tolerance. The consequence is that we may intervene with a single factor which in itself is necessary but may not be sufficient to induce tolerance. For example, if foods or micronutrients are introduced into the diet of young western infants with reduced microbial exposure, no effect may be seen, but we may be wrong to interpret this lack of effect as evidence of causal irrelevance. A western urban lifestyle is associated with numerous changes in the way foods are presented to young infants. For example, it may be important that food allergens be presented to the GI tract in the context of breast milk which contains numerous immunomodulatory factors [64]. Recent practices in infant weaning have had the consequence of separating exposure to breast milk from allergenic food proteins. Thus, a 6-month period of exclusive breastfeeding followed by a slow introduction of hypoallergenic foods followed in turn by the delayed introduction of food allergens means that in practice infants are rarely exposed to peanut or egg in the presence of breast milk. Similarly, the decline in the practice of premastication (see below) means that the infant may be deprived of numerous immunomodulatory factors that shape its response to food proteins.

Premastication May Be Important in the Development of Oral Tolerance Induction

Premastication occurs when mothers or child's caregivers chew up solid foods and feed the resulting mash to their infants. In a cohort study conducted in Thailand, it was shown that up to 17% of infants received premasticated foods as early as their 2nd week of age and 81% by 6 weeks of age [65]. In the US, Fein et al. [66] estimated from a nationally distributed sample of predominantly Caucasian infants an overall prevalence of premastication of

14%. Recently, in an extensive anthropological review of 119 cultures, it was shown that at least 30% of them practiced premastication [67].

Adult human saliva contains an array of cytokines, chemokines, antibodies and elements that includes IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TGF- β , IFN- γ , TNF- α and TNF- β , MIP-1 α and MIP-1 β , RANTES, vitamin D, iron, IgA, IgG4 and IgM [68]. Saliva contained in prechewed foods could transfer passively IL-10, TGF- β , vitamin D, secretory IgA, and IgG. Thus, premastication may provide antigen in an immunologically favorable milieu that promotes the development of oral tolerance to specific dietary antigens that prevents the development of allergies in the infant.

Premastication has declined worldwide over the past decades with transition from a traditional to modern lifestyle. Interestingly, over the same time period, there has been an emerging epidemic of allergies and autoimmune diseases, especially in the developed world [69]. Thus, this practice may have an important role in the development of oral tolerance mechanisms and the prevention of atopic and autoimmune diseases. Currently however, there is no evidence that premastication prevents the development of allergies and indeed no justification to recommend this practice which may increase the risk of infectious transmission however small from mother/caregiver to infant [70].

Conclusions

It is argued that antigen exposure through a disrupted skin barrier or through the gastrointestinal mucosa might be involved in the establishment of allergy and tolerance. Immune responses to such allergen exposures are likely to be modulated by nonspecific factors, such as gastrointestinal microflora, infectious exposure, dietary factors, immunomodulatory factors passively transferred and possibly sunlight exposure. It is hoped that interventional trials in progress and those to be conducted in the next few years will help to determine the relative contribution of these different factors and allow us to reduce the burden caused by food allergies.

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Discussion

Dr. Jones: Checking early vitamin D exposure is quite difficult unless you have got serum, so are there any data on the prevalence of sensitization in babies who have had phototherapy?

Dr. Lack: Not to my knowledge. I asked myself the same question a while ago and haven't found anything on that, but I think that's a great question. You might expect them to be very different, yes.

Dr. Papadopoulos: Children born with C-section are at increased risk for IgE sensitization. I wonder whether in the frame of the EAT study it would be useful to separate the group of children who are at highest risk for atopy by those with a genetic predisposition and those who were born with C-section to see whether there is a difference.

Dr. Lack: We intend to do that by the way, but I don't think that'll work in the LEAP study because they are all highly atopic, but in the EAT study, where we have a mixture of atopic and non-atopic, you may find differences. I personally think that there are so many confounding factors for C-section, one of them being high rate of

first-born infants, which we know is associated with the high rates of allergy, so it's very difficult to separate those apart, but we will be looking for that.

Dr. Rings: I have a question related to one of your comments on the anaphylaxis and the low incidence. You made the observation that a lot of children and young adults, are walking around with an EpiPen these days, which also gives a certain burden knowing that that's related to severe anaphylaxis. So I was wondering, if you are taking that observation into account in your EAT and LEAP studies – the risk of anaphylaxis related to food allergen. That might be something that can be lowered with the early introduction of food allergen.

Dr. Lack: The only place I alluded to carrying an EpiPen was in the vitamin D study, where that was used as a surrogate marker of food allergy in a population. In the EAT and LEAP studies we were defining food allergy by double-blind placebo-controlled food challenges at the end of the study, not by the number of children who have been prescribed an EpiPen. Obviously, if children developed allergies along the way, we gave them all the standard allergy advice, which includes carrying injectable epinephrine.

Dr. Rings: Yes, but lowering that amount of prescribed EpiPens would be a very nice end point, because it gives a certain burden, walking around with an EpiPen.

Dr. Lack: I agree, getting rid of the EpiPens, getting rid of the food allergy in the first place would be a nice end point. It's something we hope to achieve, but we will have to see what the final outcome is because of all these other factors that may come into play to induce tolerance. If we are deficient in microbes, bugs, if we are deficient in vitamin D, if we are deficient in other factors that we may even ignore at the moment, the necessary conditions for oral tolerance induction may not be there. We hope that they will, and we hope to see an effect.

Dr. Fasano: I was really impressed to see the skin barrier business, and I don't know if you are familiar with Barbara Becks's work. She just made this observation, I believe it's already been published, that apart from the stratus corneum barrier defects she also found defects in tight junction. One of the components of tight junction is downregulated, and she claimed that this is a key element to lose barrier function in atopic dermatitis, making this trafficking a key element in terms of this yin and yang between tolerance and immune response. What would you think that this would entail? And how important is indeed the route of exposure, with the skin being the one where you have to develop an immune response that leads to allergy compared to the mucosal route where you have a chance of tolerogenic response?

Dr. Lack: I think it's OK to have cutaneous exposure even if you have an inflamed skin. The theory goes that so long as you have got oral tolerance occurring at the same time, you get a balance in these two routes, and the outcome will be tolerance. I haven't presented the work that Susan Chan in our group has done, which is to look at the origin of lymphocyte, T cell responses, in allergic and tolerant children, and we have been following sensitization based on different T cell compartments in the T cell circulation. So what Susan did is she isolated skin-homing lymphocytes at the CLA-positive cutaneous lymphocyte antigen-positive lymphocytes and got very high purity, and then she isolated from the same or different patients the $\alpha_4\beta_7$, those T cells that home to the gut. What is interesting is we find in the allergic children that proliferative response to peanut is derived almost entirely from the CLA population. Now our hypothesis was that in the tolerant children it would all be in the $\alpha_4\beta_7$ but it's not, you see a mix, so you see T cells that respond, there is about a 1:1 ratio in the response rate. I think in the children with eczema who are tolerant, both routes are occurring, and there is a balance. As far as the mechanisms of decreasing skin permeability are concerned, I think it's important, but I think there are numerous others we don't know much about. Donnelly, Yung and others have shown that as eczema worsens, even in

the wild type where you have normal filaggrin expression, IL-4 and IL-5 switch off filaggrin expression in the skin. So, I think there are other mechanisms, and of course we know that IL-5 and other cytokines induce apoptosis of keratinocytes, which is why when you look microscopically at the skin in atopic dermatitis you see spongiosis, these empty gaps in the skin. So, there is a lot of things contributing to that.

Dr. Simmer: Thank you for your interesting talk. As I understand it, you allergists have come up with one recommendation for prevention which is exclusive breastfeeding for 6 months. Whereas everyone here would promote breastfeeding for 6 months for lots of reasons, you are saying that you believe breastfeeding per se reduces atopy compared with partially hydrolyzed formulas. Are you recommending breastfeeding because you are a pediatrician or are you recommending breastfeeding because you really think it reduces allergy?

Dr. Lack: There are several strands to that question. First of all, the 6 months recommendation obviously didn't come out of allergy, it came for other beneficial health effects which I think few allergists or pediatricians in their right mind would deny. So, the issue really is exclusive breastfeeding versus feeding with solids. Do I believe that breastfeeding has a protective effect? Yes, I do, but I could also cite you, and it would be very dishonest of me to do so, but I could cite you studies that show the deleterious effects of prolonged breastfeeding on the development of allergies. The problem is those studies aren't possible to interpret, especially those after the late 1970s and early 1980s. Why? Early in the 1970s, we had some studies showing protective effects of breast milk, not huge protective effects but I do believe there were some protective effects, then along came the public health message that this was a good thing to do in atopic families. What you find now in all the atopic populations, and these are observational studies, prolonged exclusive breastfeeding is practiced but that's reverse causality. That has been done because the population is advised by their GPs and pediatricians to do so because there is a family risk and you can't interpret those studies. So, I think there has been a C change in the effect we see before the late 1970s and from the mid-1980s onwards. I think it's impossible to interpret without doing randomized controlled trials, which is why we are doing particularly the EAT study to try and disentangle these effects. But I think that breastfeeding, and I am not advocating premastication either, but it was until very recently practiced very widely and still is, including the US. There are surveys showing a significant percentage of population practicing it, and in parts of Asia it's practiced as of 2 months of age, so those may be factors, breast milk, colostrum, some of the factors we see may be very important. Obviously, we feel and the ethics committee felt it was ethical to do the study we are doing, but it would be completely unethical to randomize children to breastfeeding or not breastfeeding as a randomized control study, so that study will never be done.

Dr. Klisch: A few years ago, when I first met Wesley Burks he had just identified the peanut epitope and the gene that produced it. That got me thinking about the chemistry of allergic epitopes. At the time, I recall a paper that looked at a myriad of allergic epitopes and showed that there was some commonality within their chemical structure. They seemed to fall into groups based on not only their chemical structure but their tertiary structure as well. It always seemed to me that if that science was true, it would be possible to develop treatment for allergies through vaccines, etc. Since I haven't followed that science, has it moved forward?

Dr. Lack: I think probably both of us should answer that one. That sort of links in with the question which is one of the holy grails of allergy: what makes a protein an allergen? I think we are still a long way from answering that question. There are certain properties of protein allergens that have been identified, they're more stable to heat they're more stable to digestion, they fall within a certain molecular weight, but there are numerous exceptions to this, and there are some proteins you would predict

to be allergens that very rarely cause allergies and vice versa. I think that component-resolved diagnostics, as it's now known, where you look for IgE to different allergens within peanut, are proving to be very helpful. So, there is a recent publication showing that the specificity of IgE to arah2, one of the major peanut allergens, is extremely high. If you have a level of 0.3 something, the specificity is 97%. So, if you have IgE to arah2 you nearly always have peanut allergy. So what are the properties of arah2 that make it an allergen? I don't really think we have advanced much on that front but Dr. Shreffler, you may have some observations from some of your work.

Dr. Shreffler: I think what Gideon said is on the money, that is that basic structural properties of the allergens such as the stability, size, etc., are repeatedly observed but with many exceptions. It's true that among all protein families or structural motifs, allergens are overrepresented by relative few. But if you look specifically at the epitopes on those allergens, there have not been consistent structures that emerge as being more likely to produce an IgE response versus an IgG response, to my knowledge. Where there is a lot of interest, however, and that's relatively new, is discovery of the innate immune stimulatory properties of allergens. Examples of both respiratory allergens and dietary allergens have emerged in which the allergens themselves or intimately associated molecules have the capacity to directly stimulate the innate immune system.

Learning to Prefer the Familiar in Obesogenic Environments

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Abstract

What has become familiar tends to be preferred while the unfamiliar is avoided. Additionally, liking is impacted by associative learning processes where new stimuli become liked via repeated pairings with familiar, already-liked stimuli. In addition to the ability to learn to like new foods and flavors, infants bring genetic taste predispositions to the table, including an unlearned preference for sweet and salty tastes and a tendency to reject bitter and sour tastes. When diets were plant based, unlearned preferences for sweet and salty tastes promoted intake of foods that were relatively rare in nature but were good sources of essential nutrients; the presence of the preferred basic tastes in food no longer predicts scarce nutrients. Our 'obesogenic' dietary landscape is replete with sweet and salty foods that are energy dense, inexpensive, and exquisitely tuned to our genetic taste predispositions. In the current environment, early familiarization and associative learning can result in unhealthy diets and may promote obesity risk, but we suggest applying what we know about how food liking is learned to promote healthier diets. We review classic and current evidence demonstrating how familiarization and associative learning may be used to promote the intake of initially rejected foods like vegetables within an obesogenic context.

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Familiarization in Obesogenic Environments

Through experience, things become familiar; familiarity is a natural consequence of living [1]. Learning is the process of acquiring familiarity with objects, people, actions, and their consequences. Stimuli that are familiar provide a standard against which other stimuli seem unfamiliar. The distinction between the familiar and unfamiliar is particularly important because familiarity has a powerful evaluative component; what has become familiar

tends to be liked and preferred, while the unfamiliar is disliked, avoided, or even feared. In early life, the infant learns to prefer the people, objects, and activities that become familiar through experience: he develops an attachment to his mother, preferring her to others and begins to respond with fear to unfamiliar 'strangers'. His familiar blanket provides comfort, and of particular relevance here, the milk he consumes also becomes familiar.

Initially, the infant diet consists exclusively of milk. When the transition to the adult diet begins at weaning, the child's familiarity with the flavors of milk provides the standard for determining the extent to which new foods seem unfamiliar; the more unfamiliar they seem, the less likely it is that they will be readily accepted. Formula-fed and breastfed infants differ in what constitutes the domain of the familiar. The exclusively formula-fed infant only has experience with the flavor of formula; for the breastfed infant, the familiar is more broadly defined because a variety of flavors have already become familiar. Flavors of foods consumed by the mother flavor her breast milk [2], providing a 'flavor bridge' by making the flavors of the maternal diet familiar and easing the dramatic dietary transition that occurs in the first years of life. Relative to formula-fed infants, breastfed infants showed more rapid acceptance of pureed vegetables during weaning [3]. Very early experience with flavors defines the familiar and shapes food likes and dislikes during infancy and early childhood. The importance of liking in determining the intake of infants and young children is paramount; their preferences are especially powerful predictors of intake early in life [4, 5]. Blissfully ignorant of the importance of nutrition, young children eat what they like and leave the rest.

The effects of exposure are likely to be greatest during weaning, when all solid foods are new, and acceptance of some foods is essential to consuming a diet that supports growth and health. It appears that earlier in life, when nearly all foods are new, familiarization occurs rapidly, requiring few exposures. For example, infants who were just being introduced to pureed foods increased their intake of new fruits and vegetables significantly after a single exposure, and the effects of exposure generalized to other new pureed foods [6]. The first 2 years of life are characterized by relatively weak food 'neophobia' [7] that can often be changed to acceptance with relatively limited experience [8], providing a window of opportunity for promoting acceptance of new foods. Although evidence is limited, by defining the domain of the familiar, early experience with food forms the basis for how the individual will respond to new foods, flavors, and cuisines in subsequent encounters later in life. Neophobia, the fear and avoidance of novel foods and flavors, increases and peaks in early childhood, declining thereafter. It appears that by adulthood, the impact of repeated exposure on promoting acceptance is weak [9].

Being prepared to learn to prefer the familiar allows omnivores the flexibility to come to like and eat the foods available across a wide range of environments. Infants bring additional genetic predispositions to the table, including an unlearned preference for sweet and salty tastes and an initial rejection of

bitter and sour tastes [10]. When diets were more plant based, the unlearned preference for sweet and salty tastes promoted intake of foods that were relatively rare in nature but were good sources of essential nutrients: energy and micronutrients in the case of sweet taste and essential minerals in the case of salt [11]. These days, the presence of the preferred basic tastes in food no longer predicts scarce essential nutrients. A major reason that the current food environment is obesogenic is that our dietary landscape is replete with sweet and salty foods that are palatable, pervasive, energy dense, inexpensive, extensively marketed, and exquisitely tuned to our unlearned predispositions to like these tastes. These foods will be accepted without having to become familiar. Because these readily accepted energy-dense foods are ubiquitous, they become familiar to infants and young children, while fruits and vegetables have become scarce and unfamiliar; while research is lacking, a recent documentary revealed that children were unable to correctly identify common vegetables [12].

These days, it is challenging to promote liking and intake of foods that compose healthy diets. Take vegetables, which can bring the additional challenge of having bitter taste components, further reducing the likelihood that they will be initially accepted and consumed. Support for the view that vegetables are absent in the lives of many of today's infants and toddlers comes from the results of the Feeding Infants and Toddlers Study, which showed that infants and toddlers are eating the many of the same foods that characterize the broader obesogenic environment. Approximately one third of infants and toddlers consumed no vegetables and no fruits on an average day; French fries were the most commonly consumed vegetable among 15–18 month olds, and approximately half of infants and toddlers regularly consumed desserts and sweetened beverages [13]. These patterns of intake do not conform to current nutritional guidelines for young children and reveal that diet quality begins to decline as soon as the adult diet begins to be adopted [14].

Unfortunately, the majority of parents and caregivers are not aware of the power of familiarization in determining liking early in life. Parents may offer a new food once, and if the child rejects it (likely if it is unfamiliar and not sweet or salty), the parent concludes that the child dislikes the food and does not offer it again, providing no opportunity for familiarization to occur. This approach to introducing new foods is highly successful at producing 'picky eaters' who will only consume a few foods. Providing information to parents and caregivers that the rejection of new foods is normal and adaptive and giving guidelines on how to familiarize children with new foods could be effective in reducing the likelihood of children being labeled as 'picky eaters' and in promoting the acceptance of healthy foods. Although in the current obesogenic environment early familiarization may often result in diets that fail to meet dietary recommendations and may promote excessive weight gain and obesity risk, we could apply what we know about how food liking is learned to promote familiarity with foods that comprise healthy diets.

Learning Processes Influencing Food Liking: Familiarization through Repeated Exposure

Familiarization learning is the simplest of several types of learning affecting the acquisition of food preferences and eating behaviors and results from repeated 'mere exposure'. Mennella and colleagues have demonstrated that familiarization begins prenatally; infants are more likely to accept flavors that they previously experienced in utero, during breastfeeding [2], or during formula feeding [15]. When Mennella et al. [2] provided early experience with carrot flavor either in utero or during lactation, this early familiarization affected infants' acceptance of the flavor when it was offered in infant cereal during weaning. In two experiments, Birch and Marlin [8] presented novel fruits or cheeses to 2-year-olds. The frequency with which children tasted 5 initially novel foods was varied within children, so that for each child, some foods were offered repeatedly and others remained novel. In both experiments, children's preferences for the foods increased as exposure frequency increased. Wardle et al. [16] have demonstrated that repeated exposure can also increase 2- to 6-year-olds' acceptance of an initially disliked vegetable in the home context.

Learning Processes Influencing Food Liking: Associative Conditioning

Familiarization with food and eating does not occur in a vacuum but in the context of the child's ongoing daily experiences, and other aspects of experience can become associated with foods, altering liking. Evidence for the impact of these associative learning processes on children's developing food and flavor preferences is reviewed below, organized by the stimuli that become associated with the food. These include flavor-nutrient learning, flavor-flavor learning, and flavor-context learning (table 1). Extensive research has provided evidence that these associative processes affect liking and intake in animal models [17], but the literature with humans is limited [18]. Below, we review the evidence for the familiarization and associative learning in infancy and early childhood. The common thread here is that young children learn to like (or dislike) new flavors via familiarization alone and through the formation of associations between foods and other aspects of the context of eating. These aspects include the postingestive consequences of eating the food, the addition of well-liked flavors to the food, or caregivers' feeding practices. The early learning of food likes and dislikes affects intake during key periods of early development, affecting not only growth trajectories, but also the familiar standard against which future flavor experiences are evaluated.

In associative conditioning, the individual's liking of an unfamiliar stimulus (the conditioned stimulus, or CS) changes after it is repeatedly paired with a

Table 1. Learning paradigms affecting children's food preferences

Paradigm	Definition	Example of effects
Familiarization	Repeated presentations of an unfamiliar stimulus on its own lead to increases in familiarity and liking.	When 2-year-old children were exposed to novel cheeses and fruits at different exposure frequencies, preferences increased with increased exposure frequency [8].
Flavor-consequence/ nutrient learning	An unfamiliar flavor (CS) is repeatedly paired with an energy-rich substance like sugar or fat (US). The positive valence associated with the effects of ingesting energy becomes associated with the unfamiliar flavor; liking of this flavor on its own increases.	When 2- to 5-year-old children consumed novel-flavored yogurts that were high or low in fat and energy density, they increased their preference for the flavor that had been previously paired with a high energy density [20].
Flavor-flavor learning	An unfamiliar flavor (CS) is repeatedly paired with a familiar, liked flavor such as a sweet taste (US). The positive valence associated with the taste of the already-liked flavor becomes associated with the unfamiliar flavor, so that liking of this flavor on its own increases.	When 5-year-old children were provided with two new vegetable flavors, one sweetened and one unsweetened, they developed a preference for the previously sweetened vegetable flavor [22]. (The flavors were presented in pairs, so the authors argue that the results exemplify flavor-flavor learning and not flavor-nutrient learning as both flavors were technically paired with the post-ingestive consequences of dextrose.)
Flavor-context learning	An unfamiliar flavor (CS) is repeatedly paired with a positive, social experience, such as a birthday party or praise (US). The positive valence of these experiences becomes associated with the unfamiliar flavor, so that liking of this flavor on its own increases.	Pre-school children's preferences increased for familiar snack foods repeatedly paired with adult attention or given as rewards [24].

The examples provided are those in which children learn to like healthy foods. In our current environment without intervention, it is likely that children are learning to like sweet, salty, energy-dense foods through similar processes.

familiar stimulus that already evokes a positive or negative hedonic valence (the unconditioned stimulus, or US). After repeated pairings with a positive US, the valence of the CS increases, such that presentation of the CS alone evokes greater liking than it did initially. In all of the specific paradigms that follow, the CS will be a new flavor and will be paired with a US that is liked and familiar.

Flavor-Nutrient Learning

In flavor-nutrient learning, the US is the positive consequence experienced after ingesting an energy-rich substance, such as sugar or fat. Sclafani and Nissenbaum [19] have conducted an extensive program of research to investigate flavor-nutrient learning in the rat. In the basic protocol, unfamiliar flavors (CSs) are provided. The CS flavor is paired with a US that produces a positive gastrointestinal consequence. In one series of experiments, this was an energy-rich concentrated starch solution, infused intragastrically, while another flavor was paired with infused water. When preference for the two flavors was tested after pairing with the infusions, the nutrient-paired flavor was preferred over the flavor that had been paired with water. This work inspired research in our laboratory to investigate flavor-nutrient conditioning in young children; when 2- to 5-year-old children consumed novel-flavored yogurts that were high or low in fat and energy density, they showed greater increases in liking for the high-energy-paired flavor [20]; similar findings were obtained when starch was used as the US [21]. Through most of human history, when food was scarce, learning to prefer flavors of foods that were good sources of energy was adaptive. However, in the current obesogenic context, our predisposition to learn to prefer energy-dense over energy-dilute foods may promote excessive intake, weight gain, and obesity.

Flavor-Flavor Learning

In flavor-flavor learning, the CS is an unfamiliar flavor, and the US is a familiar, already liked flavor. Through repeated pairings of the two flavors, the unfamiliar flavor becomes associated with the familiar flavor, increasing the liking of the new flavor when it is presented alone. When 5-year-old children were provided with two new vegetable flavors in liquid form, one sweetened and one unsweetened, they developed a preference for the previously sweetened vegetable flavor [22].

We recently compared the effects of 'mere exposure' familiarization with flavor-flavor associative conditioning on 3- to 5-year-old children's vegetable liking and intake. The 12-week procedure took place in children's regular day-care center classrooms and involved pre-tests assessing liking and intake of vegetables, ten exposure trials, and post-tests assessing liking and intake. To assess liking, children tasted five cooked vegetables (broccoli, cauliflower, red peppers, sugar snap peas, and yellow squash) and rated them [4]. During the exposure trials, the children were asked to taste a very small sample (about

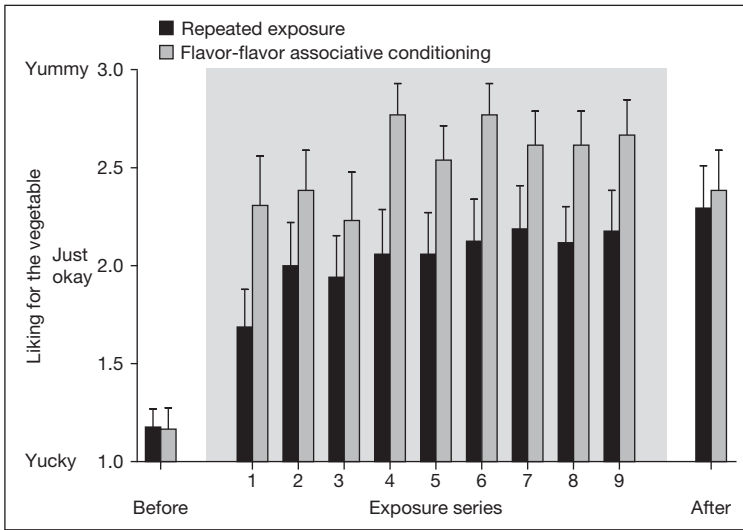


Fig. 1. Repeated exposure and flavor-flavor associative conditioning increase vegetable liking. In both conditions, there was a significant difference between liking in the first and second tasting exposures ($p < .05$), and increases in liking were maintained throughout the experiment. Note: 30 children who found vegetable to be yummy during pre-exposure were excluded. Results were similar with the total sample.

4 g) of a vegetable (yellow squash or red bell pepper) that was generally unfamiliar and rated as either 'yucky' or 'just okay' by most children. Children assigned to the repeated exposure condition tasted and rated the vegetable during each of the exposure trials. Children assigned to associative conditioning were given a small amount of a flavored dip that they thought was 'yummy' and dipped the vegetable into it prior to tasting during each exposure trial; dip options included ketchup, ranch, and cinnamon sugar [23].

Although we hypothesized that associating a liked flavor with the vegetable would produce greater increases in liking and intake, both familiarization and flavor-flavor associative conditioning produced the same significant increases in liking and intake, as shown in figure 1. Increases in liking occurred rapidly with a significant increase in vegetable liking between the first and second tasting exposures. In both conditions, liking for the vegetable that was repeatedly tasted, either with or without dip, was significantly greater than for the other vegetables that had not been tasted repeatedly. From before to after vegetable exposure, intake increased dramatically, more than doubling, from 15 to 33 g. These initial findings reveal that repeated exposure and associative conditioning had similar, positive effects on liking and intake of initially disliked vegetables, and that these effects occurred with only a few brief taste exposures [23].

Because this experiment was conducted in the context of children's preschool program, the findings suggest that such approaches could be readily adapted for use as part of ongoing activities in preschool settings.

Social Influences

Liking for an unfamiliar flavor can also increase when the flavor is repeatedly paired with positive social experiences. This type of conditioning can take many forms. For example, children's preferences increased when sweet and non-sweet snack foods were paired with positive adult attention or given as rewards [24]. There is also evidence that learned liking for foods served in positive social contexts can generalize to other, similar foods [25]. Overall, experimental studies demonstrate that young children learn to like flavors that become familiar through familiarization and through repeated association of foods with already-liked stimuli.

Evidence for Persistent Effects of Early Familiarization

We have argued that early experience is important because it defines the familiar, and the familiar tends to be liked, preferred, and consumed and shapes the child's subsequent responses to new foods. This is particularly important for children, who eat what they like and leave the rest. Mennella's work has revealed that flavor experience in utero, during lactation, and during formula feeding affects the infant's subsequent response to complementary foods. In a recent intervention we conducted to prevent excessive weight gain during the first year of life, a component of the intervention focused on the transition to solids. Intervention group mothers received guidance on promoting the acceptance of new complementary foods [26]. When mothers had begun to introduce infant cereal successfully and infants were 4–6 months old, four commercially available pureed vegetables were provided (Gerber pureed green beans, peas, squash, and carrots). One vegetable was offered each week for 6 consecutive days. Intake was measured before and after repeated exposure. Infants in this intervention group significantly increased their intake of green beans, peas, and squash from the first to the 6th day of exposure. Carrot intake was already high prior to exposure, probably due to carrots' sweeter taste and/or generalization from the other vegetables as carrots were offered last [26, 27]. We assessed the longer-term effects of this intervention on infants' acceptance of new foods when infants were one year old by having mothers offer their infant an unfamiliar food (hummus, cottage cheese, or yogurt); infants' responses were videotaped and coded. Fewer intervention infants rejected the unfamiliar food at one year (10%), compared to the control group (25%) [27]. This finding suggests long-term effects of early repeated exposure on subsequent acceptance of novel foods. Repeated exposure to healthy foods may also impact early growth and weight status given that our interventions affected infant weight status at one year [26].

Conclusions

Familiarization and associative conditioning processes can increase liking and intake of foods and flavors. By shaping what is familiar and preferred, these processes have lasting effects on intake and weight status. Early experience with food provides the initial basis for delineating the familiar from the unfamiliar. At weaning, all foods are new, and early experiences with food can either limit or expand the boundaries of the familiar. If early experience includes exposure to a variety of foods and flavors, then a wide range of foods and flavors will be accepted. However, in order for this to happen, caregivers need information: that the rejection of new foods is normal, and that familiarization can be used to promote children's liking of healthy foods. Without this guidance, it is likely that their diets will be dominated by sweet or salty foods that are readily accepted without familiarization. In contexts of food scarcity, acceptance of only a few foods during weaning can result in inadequate growth, but in our obesogenic environment, limited variety can result in diets too high in added sugar, salt, and energy and too low in dietary variety, fruits and vegetables, fiber, and micronutrients [13].

The familiar continues to be redefined through our daily encounters with food and eating, although it appears that early experience is key in shaping our subsequent encounters with new foods and cuisines. Outside of the laboratory, eating episodes are complex and are accompanied by many positive and negative contextual stimuli that can be associated with food and can modify the effects of familiarization. Early experience provides a basis for food likes and dislikes, shaping subsequent patterns of dietary intake and obesity risk. The practical implication is that children should have frequent early experience with a variety of foods that comprise healthy diets. This experience may be most effective in promoting liking and acceptance if it occurs in association with already-liked flavors and foods, in positive social contexts.

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Discussion

Dr. Villalpando: I work with school children and adolescents who are obese, and we are sure that at least in Mexico these kids are not eating enough vegetables. Is there a decisive point during their growth that makes them reject later on certain types of foods or colors of food?

Dr. Birch: I think many of you who are parents would probably agree that adolescence is a time when kids are rejecting a lot of things that are connected with the adult establishment. Still, although evidence is limited, it appears that some of their negative reactions to 'healthy' foods are temporary and they will return to earlier patterns of preference later on. In addition, by the time children reach adolescence, they have established patterns of intake. Because it is easier to shape new behaviors than to alter existing ones, changing adolescents' food intake patterns is particularly

challenging. There is research in the US looking at the effects of involving children and adolescents with gardening, growing, and cooking that has shown some positive effects. This requires a lot of effort, time and resources. Other approaches to making healthy foods available and more affordable have also shown promise.

Dr. Papadopoulou: We know from several studies that taste preferences of the children are established very early in life. You mentioned breastfeeding. I am thinking about the early weaning period. The usual practice is to introduce cereals with some sweet taste to infants as the first weaning foods. If we switch to vegetable puree as the first weaning food and introduce sweet foods after the infants have been exposed to a variety of vegetables and fruits, will this have an impact on children's taste preferences and dietary habits in the long term?

Dr. Birch: I think there might be as many different opinions as there are people in this room about that, but since you ask me I would say that again we don't have much of an evidence base for deciding this. Infants will like and eat sweet things no matter when they are introduced, and certainly I think your hypothesis is a reasonable one; namely if the infant first becomes familiar with sweet foods, this may make it less likely that most vegetables will be readily accepted, because they lack the familiar sweet taste. Dr. Mennella has done research that can speak to your question.

Dr. Mennella: We have done some work where we found that having prior experience with fruits didn't interfere with vegetable acceptance, but once the baby following from your own work has repeated exposure to vegetables will lead to increased acceptance of variety. But I think that one key point is that for those foods that taste bitter the experience has to be with bitter taste in food. So, you can give a variety of fruits, it's not going to impact on green vegetables. The baby has to become familiar with that bitter taste in order to learn to like it.

Dr. Birch: There is some generalization, there is no generalization across bitter.

Dr. Were: It is very interesting that we can observe and determine children's taste and how they move on to prefer those later. Let's get back to the children we are treating and obesity which is an issue that is concerning us, including me and the country I come from. Can we use this information to deliberately give foods that we know these babies don't like and reduce the foods that they like in order to influence their body mass accretion? It is probably an ethical dilemma whether we can deliberately remove a food that we know a child would like and observe the child eat less because they don't like what we are giving them. How do you see this experience?

Dr. Birch: I am suggesting that we could use what we know about how kids learn during this early period to promote acceptance and liking of foods that we think they should be eating. That would improve diet quality, foods that are lower in energy density that might be more consistent with diet so they are not going to promote obesity. However, as was pointed out by a previous speaker, it's often easy to understand what needs to happen, but it's much harder to know how to get it done. For example, we have noted that familiarization leads to increased acceptance and intake of foods by children, but when we have tried to conduct research to familiarize children with fruits and vegetables in child care settings, caregivers can be a major barrier: they often refuse to eat the food. So they are modeling the very behaviors we are trying to avoid in the children. Unfortunately, if we can't change the adults' diets we will have trouble changing children's diets. However, I think child care settings do provide many opportunities to familiarize children with healthy foods that they may not have access to, and may not be eating at home.

Dr. Stettler: What you presented about vegetables is consistent with the observation that kids don't eat enough vegetables. But I have more difficulty in reconciling your findings with children's fruit intakes because fruits are sweet so you would predict that kids would eat more rather than less than what is recommended. So why is

that not the case? Do fruits need to be combined with an energy-dense product to make them acceptable? And why is fruit intake not higher since they are sweet?

Dr. Birch: So why isn't fruit intake higher? I think fruit intake is less of a problem than vegetable intake. I think it still is, in many cases it's just a matter of availability and accessibility, at least for certain populations. We know that in many low-income areas, there are few supermarkets or other outlets available where you can buy fresh fruits and vegetables; they are expensive and have a short shelf life, and they don't tend to be offered in fast food places. So, I think it's more a problem of availability than potential acceptability of them.

Dr. Siega-Riz: I also think that it happens to do with the cost of fruits and vegetables and also the fact that mothers think that if they give juice to their children and they just drink all the juice, then in fact they are getting their fruits.

Dr. Gottrand: My question is related to the type of taste for the same food. For example, if you take a carrot, a manufactured product from the industry doesn't have the same taste than a carrot coming directly from the garden. Do you have any data comparing for the same fruit or vegetable if the taste is strong, sweet or acidic, if it can change something in the acceptance of the infant, or if it's better to start with smoother, more harmonized taste rather than strong, acidic or sweet for later acceptance of the same food?

Dr. Birch: I am not sure we know the answer to that one. I think that what we often see as subtle differences or trivial differences in food preparation can have a powerful impact on children's willingness to eat a familiar food, or to recognize a food as familiar. Dr. Mennella, do you have any comments about that in terms of your work?

Dr. Mennella: There has really been no good experimental research that looks at different quality of a vegetable and what the taste dimension is in acceptance. But what I would say is that the evidence seems to suggest that children will come to prefer that which they are fed, and so one way of thinking about it is we teach a child what it should taste like and so what children are offered is what they eat.

Dr. Birch: Children begin to learn early in life about what is appropriate based on how foods are prepared and served to them. We did some research on children's acceptance of tofu years ago. Some of the children were served tofu with nothing added, some had it with sugar added, while a third group were served tofu with salt added. Results showed that children learned to prefer and eat it tofu the way it was served to them. If they had been given tofu without added sugar or salt, that was the way they preferred it, suggesting that they are learning some simple cuisine rules about whether is it 'appropriate' to add sugar or salt, and that these rules emerged from how the food was prepared and served to them.

Dr. Klish: I found your longitudinal studies interesting. When you stratified the children based on sugar intake or sweetened beverage intake, you saw differences in body mass index that persisted out to age 15 years. Have you analyzed your data on the intake of fruits and vegetables in the same way?

Dr. Birch: We haven't done that research.

Dr. Klish: The reason I ask the question is: there is much focus on fruits and vegetables, particularly in our schools, which puts a strain on school food budgets. I am not sure I have seen data that show a relationship to BMI over time.

Dr. Birch: I think the data are complicated and incomplete in adults.

Dr. Agarwal: In India, children who reached the age of 2.5 years go to playschool, and we tell the teachers what children should eat as right snacks. In turn, the teacher sends a message to the families to give green vegetables and mixed cereal snacks to kids (this goes on for 5 days in a week). School child nutrition message teaches the family about nutrition and dietetics.

Dr. Birch: Your example makes a very important point; the child's experience with food in out of home settings such as school or childcare provides opportunities for children to serve as change agents in the family. In the US, it is a major challenge to find effective ways to reach families in order to make dietary change.

Dr. Puri: The influence on food patterns in childhood is not only restricted to taste. There are so many other sociocultural issues to this; it is not just a direct simplistic relationship. Parenting styles have a strong influence. If the parents are more permissive, the preference for sodas and unhealthy foods is higher in these young children. If the parenting styles are more disciplinarian, the preferences for fruits and vegetables and healthier food options become greater.

Dr. Birch: I think you are absolutely right, and parenting styles and parents' feeding practices are really incredibly important. This is another area where parents need anticipatory guidance because childhood obesity can be viewed as a new threat to children's health, one that requires very different parenting responses than the challenges posed by food scarcity.

Dr. Madrazo: My comment goes in the same way. In my practice, I see parents who do not pay much attention to what their kids eat. They just buy what they consider the right food, put the food on the table and the kids will eat it. Those kids are the ones who do better. They don't have problems of obesity. Their problems are not related to food. Aren't we paying too much attention as parents or health professionals to those issues, and are we trying to force the kids to eat the right food? We were all children and we didn't like vegetables, and now we eat vegetables as adults. I understand now obesity and feeding disorders are important problems in pediatrics. Maybe by trying different strategies like in the past we would achieve better results.

Dr. Birch: I think that's a very important point. I haven't said much about this today, but we have done a lot of research on the effects of parents' feeding practices; for example, we find that parents who restrict access to palatable foods have children who tend to overeat those foods when they are available. One challenge is to find ways to help parents to deal effectively with their concerns, while helping them to promote their children's acceptance and liking of healthy foods.

Dr. Siega-Riz: You also can't deny the fact that we are now in an environment that has more food, has more processed foods and people are eating out more. So in the past when we used to eat out it was a treat, and now it's an every day occurrence. So, there is plenty of evidence that has actually shown that these dietary patterns over time are negatively influencing our health status. So we do need to retrain how we are eating

Dr. Guandalini: In longitudinal studies such as those on girls 5–15 years of age, other variables should perhaps be considered. I wonder, for instance, whether physical activity was taken into account as part of the lifestyle. Does physical activity play any role in dietary choices and in influencing eventual body mass index?

Dr. Birch: We do have accelerometry data on these girls, so the question you raise is one that we have tried to address. However, we haven't seen clear relations between accelerometry measures of physical activity and girls' weight status. But in our data, we did see that parents' eating and activity patterns were consistently related. That is, parents who were more physically active and had healthier diets tended to have daughters with lower weight status. In contrast, relative to this group, parents of heavier girls had lower levels of physical activity and consumed diets that were higher in energy. So the latter set of families could be characterized as 'obesogenic'. Unfortunately, it isn't possible to establish cause and effect or to tease apart effects of diet and activity on weight status in this observational study.

Early Feeding Practices and Their Impact on Development of Celiac Disease

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Abstract

Celiac disease is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. Gluten is a protein component in wheat and other cereals, including rye and barley that are generally introduced in the infant's diet at weaning. At present, two schools of thought claim that changing early feeding regimens in at-risk infants can either prevent the onset of the disease or merely delay it. Recent advances have increased our understanding of the molecular basis of this disorder and provide the rationale to perform prospective dietary interventional studies to establish the proper timing of gluten exposure to minimize the risk of developing celiac disease.

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Introduction

Celiac disease (CD), or gluten-sensitive enteropathy, is an immune-mediated chronic enteropathy with a wide range of presenting manifestations of variable severity. It is triggered by the ingestion of gliadin fraction of wheat gluten and similar alcohol-soluble proteins (prolamines) of barley and rye in genetically susceptible subjects with subsequent immune reaction leading to small bowel inflammation and normalization of the villous architecture in response to a gluten-free diet. CD not only affects the gut, but it is a systemic disease that may cause injury to the skin, liver, joints, brain, heart, and other organs. It is a complex genetic disorder, and HLA status appears to be the strongest genetic determinant of risk for celiac autoimmunity. There

is a propensity for individuals with CD to carry specific HLA class II alleles, which have been estimated to account for up to 40% of the genetic load [1]. In affected individuals, 95% have either DQ2 (*HLA-DQA1*05-DQB1*02*) or DQ8 (*HLA-DQA1*03-DQB1*0302*), in contrast to the general population in which 39.5% have either DQ2 or DQ8 [2].

CD is now considered to be a T cell-mediated, chronic inflammatory disorder with an autoimmune component. Altered processing by intraluminal enzymes, changes in intestinal permeability, and activation of innate immunity mechanisms seem to precede the activation of the adaptive immune response [3]. It is the interplay between genes (both HLA and non-HLA associated) and environment (i.e. gluten) that leads to the intestinal damage typical of the disease [4]. Under physiological circumstances, this interplay is prevented by competent intercellular tight junctions, structures that limit the passage of macromolecules (including gluten) across the intestinal epithelial barrier. Recent evidence suggests that the gluten-induced upregulation of zonulin, a recently described intestinal peptide involved in tight junction regulation, is responsible, at least in part, for the aberrant increase in gut permeability characteristic of the early phase of CD [4] and the subsequent abnormal passage of gluten into the lamina propria. Here, the protein is deamidated by tissue transglutaminase and is then recognized by HLA-DQ2/DQ8-bearing antigen-presenting cells, thereby triggering the onset of the CD autoimmune reaction [3].

Epidemiology

The epidemiology of CD has been entirely rewritten during these last decades [5–8]. In the past, CD was considered a rare disorder, mostly affecting individuals of European origin, usually characterized by onset during the first years of life. By the way, this paradigm is still widely diffused, to the extent that in many European countries CD is included in the list of rare disorders protected by specific regulations of the health care system. On the other hand, a large number of studies have recently shown that CD is one of the commonest, lifelong disorders affecting mankind all over the world (with some remarkable exceptions). Currently, most cases remain undiagnosed, due to lack of typical symptoms, and can be recognized only through serological screening by sensitive tools, e.g. serum anti-transglutaminase determination. CD is not only frequent in developed countries, but is increasingly found in areas of the developing world, such as North Africa and India. CD can contribute substantially to childhood morbidity and mortality in many developing countries.

Nowadays, the role of epidemiology has gone well beyond the mere measurement of CD occurrence. A number of situations have been found to predispose to CD, e.g. family history of disease, associated autoimmune disorders

or Down syndrome. The characterization of these at-risk factors is not only important for diagnostic purposes, but also sheds light upon CD pathophysiology and prevention. Furthermore, epidemiological studies have highlighted the role of infant nutrition, particularly age at introduction of cereals and amount of ingested gluten, in the predisposition to CD development. This knowledge could bear implications for human nutrition at large.

Natural History of Celiac Disease

Recent reports suggested that the prevalence of CD in Western countries has been increasing during the past few decades. Rubio-Tapia et al. [9] found in adult American men a CD serology-positive prevalence of 0.2% in a cohort enrolled between 1948 and 1954 and ~0.9–1% in a different cohort enrolled recently. The same trend has been noted in Finland, where the overall prevalence of CD in two different population-based samples increased from 1.05% in 1978–1980 to 1.99% in 2000–2001 [10]. We have recently reported the true natural history of CD autoimmunity in the US. Our data demonstrated that within an American adult population CD prevalence doubled between 1974 (1 every 501 subjects) and 1989 (1 every 221 subjects) [11]. This trend was further validated by our epidemiological results in a different adult sample screened in 2001, in which we detected a CD prevalence of 1:105, suggesting that during the past 27 years the prevalence of CD among adults in the US increased 5-fold, doubling approximately every 15 years [11].

Before this study, the natural history of gluten sensitization in subjects belonging to the general population was unclear. An age-related increase in the prevalence of celiac autoimmunity had only been observed in at-risk individuals, e.g. subjects with a family history of CD or type 1 diabetes [12–15]. It has been speculated that loss of gluten tolerance leading to immunological and mucosal changes typical of CD usually develops early in life, soon after the exposure to the environmental trigger (i.e. at weaning), while the onset of clinical manifestations of the disease can manifest much later [5]. Conversely, our study demonstrated that loss of gluten tolerance may occur at any time in life for reasons that are currently unclear.

A steady rise in the incidence of autoimmune diseases as well as allergic diseases has been registered in industrialized countries over the last few decades. Both in Europe and the US, type 1 diabetes showed a stable and relatively low incidence over the first half of the 20th century, followed by a sharp increase that began some time after the middle of the century [16]. According to the hygiene hypothesis, an early childhood infection or the establishment of mixed intestinal microbiota could downregulate immunity and suppress different autoimmune disorders [17]. However, the raising prevalence of adulthood onset of CD that we observed in our study can be hardly explained by hygienic changes occurring in childhood. Our prospective cohort study

in which the same subjects were followed over time also excluded changes in the genetic component as the cause of increased prevalence of CD that we observed since 1974. Therefore, our data provide the undisputable proof that subjects genetically predisposed to CD can lose tolerance to gluten at any age. The amount and the quality of ingested gluten, type and duration of wheat dough fermentation, the spectrum of intestinal microbiota and its changes over time, enteric infections, and stressors in general are all possible switches of the tolerance/immune response balance [8, 18, 19]. However, further studies are required to clarify the relevance of these factors in causing loss of gluten tolerance and possible intervention on these factors to prevent the onset of CD and, possibly, other autoimmune diseases in genetically predisposed subjects.

Role of Early Feeding Practice in the Onset of Celiac Disease

Epidemiological data support the hypothesis that early feeding practices may influence the risk of CD development. In Sweden, an epidemic of early-onset, typical cases of CD, was observed during the 1984–1996 period. The incidence rate of symptomatic CD in children younger than 2 years of age increased 4-fold within a few years and declined in an equally abrupt manner about one decade later. The epidemic was partly explained by changes in infant feeding [20, 21]. Factors possibly influencing the disease risk were (1) duration of breastfeeding, (2) age at gluten introduction, and (3) type and amount of gluten introduced during the second semester of life.

The effect of breastfeeding on CD risk has been recently reviewed by meta-analysis of available studies. It was found that children being breastfed at the time of gluten introduction had a 52% reduction in the risk of developing CD compared with their peers who were not breastfed at the time of gluten introduction. It is biologically likely that the presence of breast milk at the time gluten is introduced increases the chance of developing oral tolerance for the major gluten antigens. An association between increasing duration of breastfeeding and reduced risk of CD was also documented. It remains unclear whether breastfeeding provides a permanent protection against CD or whether the practice only delays the onset of symptoms [22]. The mechanisms through which breast milk protects against the development of CD could include: (a) reduction in gluten intake, (b) prevention of gastrointestinal infections, and (c) protection conferred by human milk factors, e.g. secretory IgA, stimulating maturation of the intestinal barrier and downregulation of inflammatory immune responses.

The relationship between timing of gluten introduction and CD risk is still controversial. This issue was recently investigated in a prospective, observational study conducted in Denver, Colo., USA, on 1,560 children at increased risk for CD or type 1 diabetes. Children exposed to gluten-containing cere-

als in the first 3 months of life had a 5-fold increased risk of celiac serum autoimmunity compared with children exposed to gluten-containing foods at 4–6 months. Children not exposed to gluten until the 7th month or later had a marginally increased risk of celiac serum autoimmunity compared with those exposed at 4–6 months (HR, 1.87; 95% CI, 0.97–3.60). Based on these results, authors suggested that a favorable ‘window of exposure’ to gluten exists between 4 and 6 months. Outside of this period, gluten introduction may increase CD autoimmunity risk in susceptible children [23]. The ‘tolerance window’ hypothesis has been incorporated in the recent recommendations on complementary feeding formulated by the ESPGHAN Committee on nutrition. According to this group of experts, it is prudent to avoid both early (<4 months) and late ≥ 7 months) introduction of gluten and to introduce gluten gradually while the infant is still breastfed because this may reduce the risk of CD, type 1 diabetes mellitus, and wheat allergy [24]. However, the ‘gluten tolerance window’ hypothesis has not found confirmation in a similar prospective study performed on 1,511 genetically at-risk German infants. Neither the breastfeeding pattern nor the introduction of formula milk and gluten-containing or gluten-free solid food supplements during the first 3 months of life was associated with an increased risk of CD serum antibodies in this German study [25]. Antigen avoidance is a widely used tool for primary prevention of allergic disorders in children [26]. The human intestine shows a postnatal developmental pattern of the intestinal barrier function that resembles gut closure observed in other mammals [27]. However, the possibility that delayed gluten introduction may reduce the risk of CD development has never been prospectively investigated.

Finally, the previously mentioned epidemic of CD among Swedish children observed in the mid-1980s also suggested that the amount of gluten ingested during weaning can play a pivotal role in the development of CD. The regional differences in the epidemiology of CD in India also give support to the hypothesis that the amount of gluten plays an important role in the onset of CD. CD is reported frequently in high wheat-consuming states in northern India and quite rarely in the southern States, where rice is the staple food [28].

Intervention in the Infant Dietary Pattern to Change the Risk of Celiac Disease

As previously summarized, several retrospective studies have suggested that the time of gluten introduction in the diet of infants at risk for CD may affect the incidence of the disease. However, the data supporting this hypothesis are circumstantial, limited by their retrospective design, and often criticized by alternative interpretations suggesting that the delay in gluten exposure merely postpones the onset of symptoms rather than preventing the disease. Due to the cross-sectional design of these studies, it remains

unclear whether the reported microbial associations (see below) are pathogenic or merely the consequence of CD intestinal inflammation. In order to clarify the role of infant nutrition on the risk of CD development, at least two prospective intervention studies have recently been initiated. The results of these long-term studies will be available in the next years.

The Family Study of PREVENTCD

This study is currently performed in 10 European countries, and a total of 1,000 children will be involved. The participating children and mothers will be followed for a period of 1–3 years. The project will study the influence of the dietary history on the prevention of CD. The general concept is that small amounts of food substances are administered gradually to ‘teach’ the immune system not to respond to this foodstuff. This is also called ‘desensitization’ or ‘induction of tolerance’. Newborns from family at risk of CD that are exclusively breastfed and HLA-DQ2 or DQ8 positive are given 100 mg of gluten between 4 and 6 months of age. After 6 months of age, gluten is gradually introduced into their diet. CD autoantibodies are then monitored every 3–6 months to disclose gluten sensitization. The current status of PREVENTCD is that recruitment of the 1,000 infants has been concluded and now longitudinal observation and analysis are ongoing.

The Italian Baby Study

This is another initiative aimed at evaluating the role of (a) age at gluten introduction in CD-related autoimmune serological changes in a large cohort of at-risk infants (first-degree relatives of patients with CD); (b) other early environmental factors, particularly milk feeding; (c) different HLA-DQ2/DQ8 genotypes (high risk vs. low risk) in CD predisposition, and their interplay with infant nutrition patterns.

Between October 2004 and June 2007, 722 infants (51% male) at increased risk for CD were enrolled in this prospective, multicenter intervention study conducted in Italy. At weaning, gluten was introduced in a blind manner in the infants’ diet either between the 4th and 6th month (group A) or after the 12th month (group B), then the infants were followed up for 5 years. Diet (duration of breastfeeding and types of formulae, adherence to the dietary plan, amount of gluten ingested) and clinical data were collected during telephone or face-to-face interviews at 4, 7, 9 and 12 months of age. CD serology (IgA anti-transglutaminase antibodies) was tested at 15 (plus HLA-DQ genotype), 24, 36 and 60 months of age. Small intestinal biopsy was recommended in all infants that had positive CD serological tests (fig. 1). At the last study update (October 2008), duration of follow-up was at least 15 months in 100%, 15–24 months in 93%, 24–36 months in 81% and longer than 36 months in 48%. Fifty-two percent of infants were enrolled in group A and 48% in group B. Prevalence of biopsy-proven CD at 36 months was 8% in group A and 2% in group B ($p < 0.01$). At 3 years of age, the proportion of infants developing

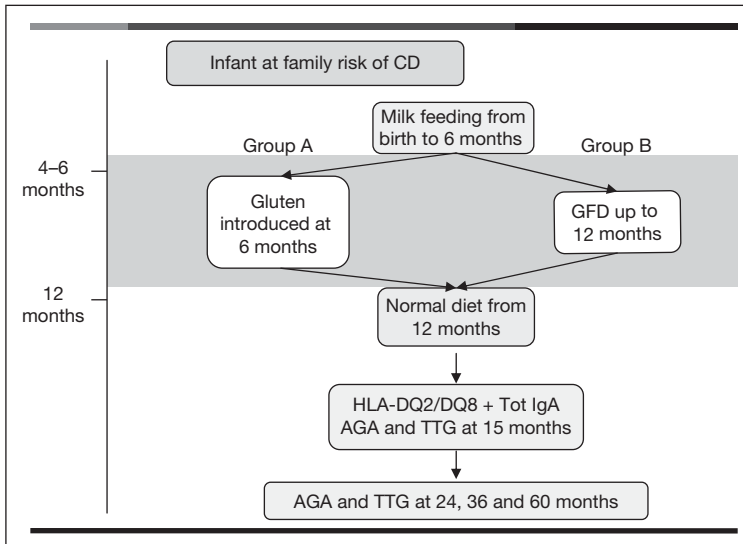


Fig. 1. Study design of the Italian baby study. GFD = Gluten-free diet; TTG = IgA anti-transglutaminase antibody; AGA = IgG anti gliadin antibodies.

biopsy-proven CD was significantly higher among those weaned with gluten at 6 than at 12 months of age. A longer follow-up is required to clarify whether the delayed gluten introduction effectively protects from CD development or merely delays the onset of the disease.

Intestinal Microbiota and Onset of Celiac Disease

One follow-up study of intestinal colonization process of the microbiota was conducted in 20 Swedish children stratified according to the genetic risk of developing CD. Total bacterial proportions were significantly higher in the high and intermediate genetic risk group than in the low genetic risk group. Gram-negative bacteria and *Bacteroides-Prevotella* proportions were higher in the high genetic risk group than in the intermediate and low genetic risk groups. In this study, the analysis of the fecal microbiota was conducted by fluorescence in situ hybridization and flow cytometry [20]. Both phenotypic methods present a substantial amount of variability and may rely on an individual and subjective interpretation, while the 16S rDNA sequencing, based on ribosomal SSU species-specific variability, has become the qualitative reference technique for bacterial taxonomy and identification [21].

In healthy infants, as described by Palmer et al. [29], *Bacteroides* colonize and establish in the GI tract. Although the timing of their first appearance varies from baby to baby, they are consistently present in nearly all infants by 24 months. The healthy microbiota evolves during different life stages, and in infants shows a lower ratio of *Firmicutes* to *Bacteroides* than in adults. Overall, the microbial ecosystems in each healthy baby achieve stability converging toward a profile characteristic of the adult GI tract in the first year of life [22]. Conversely, our recent prospective studies on the gut microbiome of infants at risk for CD suggest that their microbial ecosystem is different than that of nonpredisposed children [Ravel and Fasano, pers. commun.]. Our studies revealed that the colonization process is very dynamic, with high degree of intersubject variation over time. Unlike in nonpredisposed children, the gastrointestinal microbiota of infants at risk for CD does not stabilize towards an adult-like microbiota. Members of the phylum *Bacteroides* are absent from the GI microbiota for up to 24 months, while they are predominant in nonpredisposed children. These data suggest that early dietary and/or probiotic interventions may potentially stabilize the gut microbiota of these at-risk children, and so prevent and/or delay the onset of CD.

Conclusions

Given the undisputable role of gluten in causing inflammation and immune-mediated tissue damage, CD represents a unique model of autoimmunity in which, in contrast to most other autoimmune diseases, a close genetic association with HLA genes (DQ2 and/or DQ8), a highly specific humoral autoimmune response (autoantibodies to tissue transglutaminase), and, most importantly, the triggering environmental factor (gluten), are known. This information provides the rationale for the treatment of the disease (gluten-free diet) and for preventive interventions based on changes in early feeding practices or changes in gut microbiota. Large, multicenter dietary interventional studies and long follow-ups are necessary to generate proper evidence to change current dietary guidelines.

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Discussion

Dr. Szajewska: You kindly mentioned the PREVENTCD study funded by the EU. I just want to clarify that it's not an open study; it's a double-blind placebo-controlled study. As in your study, children are screened for HLA DQ2/DQ8, and only those children are included in our project. The recruitment phase is now finalized. We recruited more than 1,000 infants, but of course we are still waiting for the results.

Dr. Fasano: I appreciate that; I took the information from www.clinicaltrials.gov, so you may want to make some clarification there. I didn't know that PREVENTCD was a double-blind study. Thank you for the clarification.

Dr. Saavedra: Obviously, the intestine is by far the largest microbiome we have. But we also have skin flora or microbiota, and we have respiratory microbiota. We have these epithelial surfaces that are also in contact with the environment. As we heard in the concerns regarding dermal exposure or respiratory exposure, do you think that the mucosal lymphoid system both in the gut and respiratory tract have the same role, have different roles, do they depend on the allergens, do they work the same?

Dr. Fasano: You are right by saying that there are several interfaces with the environment, each having its complex microbiome. Again, just compare the lung with the gut. The lung has a uniform response, whatever is there needs to go away, nothing is supposed to be there. The gut doesn't have that luxury, it can't do that. The gut immune system has this unbelievable discriminating capability to decide what stays and what goes, not only just nutrients and microorganisms but also within microorganisms the gut immune system and pattern recognition receptors are able to distinguish between friends (commensals) and foes (potential pathogens). Nevertheless, I totally agree with you that skin and lung microbiota may play a tremendous role in antigen trafficking and, therefore, in the balance between tolerance and immune response.

Dr. Saavedra: My other concern has to do with the nice drawing that you showed at the beginning which shows one clean side of the tube and the other not so clean side of the tube. Unfortunately, all we're studying is the end of the tube. We know we have microbiota in the small bowel, and going back to what you said about what has changed to the environment, we only started pasteurizing food a hundred years ago. We today eat some fermented food, and we do a small bowel biopsy, and we get an aspirate of 10^6 CFU, that's bacterial overgrowth, that's abnormal, which probably wasn't just a hundred years ago. But we are not studying the microbiome with the small bowel, and if you look at, for example, where probiotics work more with rotavirus diarrhea, those are small bowel pathogens, not colonic pathogens. How do we study the largest, longest part of the bowel?

Dr. Fasano: I absolutely approve the questions that have been formulated by the NIH when it put in motion this project on defining the human microbiota in the GI tract. I can go even further by saying contrary to the general wisdom we now know that there is a microbiota in the stomach. We always thought it was hard for microorganisms to survive in such harsh environment. Now we realize that this is not true. What is interesting is the concept that the intestine is not like Las Vegas: what happens in the gut (or one its segments) does not stay in the gut (or in that particular segment). In other words, interactions occurring in the colon affect big time the way stuff is handled in the small intestine all the way up to the duodenum. There are several studies showing that an inflammatory process in the colon can affect functions in the small intestine and vice versa. Of course, when you have a rotavirus infection that typically affects the proximal small bowel, your colonic microbiota will change. The key question we asked ourselves when we embarked in the gut microbiota project was the relevance of the gut microbiota in the stools as representative of the colonizing microbiota in the GI tract. One of the concerns was that microorganisms in the

stool could be less efficient colonizers that are more likely eliminated with the stools and, therefore, overrepresented in stool specimens as compared to the composition of microbiota resident on the gut mucosa. This issue was put at rest when microbiota on intestinal biopsies was compared to stool microbiota and they resulted quite similar in their composition. But the most important challenge that in my opinion will also offer unbelievable opportunities is the magnitude of the task that we have on the table. Don't forget that we had to invest many years and millions of dollars to resolve the human genome made by only 30,000 genes. With the microbiome we are dealing with 100 times more genes. What we are able to do now was unthinkable a year ago; in other words, if you would be so kind to give me your stools, in 3 days and for approximately 70 dollars I will give you back the full composition of your gut microbiota. This was unthinkable until recently. So, I am assuming that something is going to happen in terms of mapping out throughout the GI tract the entire microbiota and how nutrition can impact its composition (nutrigenomics) and, therefore, the interplay between our genes and the genes of bacteria living within our GI tract. That I believe will be the most fascinating frontier.

Dr. Villalpando: I was again intrigued by microbiota because the slides show that kids with celiac disease have more Firmicutes and less protobacteria and *Bacteroides* which are the most abundant Gram-positive bacteria in the gut. Do you think it would be possible to supplement children with celiac disease with prebiotics or probiotics in an attempt to reinforce *Bacteroides* or something to deal with antibiotics?

Dr. Fasano: There are two aspects to be clarified here. One is that (and here I am purely speculative) we have witnessed this recent epidemic of autoimmune diseases because of the use and abuse of antibiotics that have tremendously impacted the gut microbiota composition in a negative and sustained (as recently shown by several studies) way. This hypothesis reconciles with the observation that the autoimmune epidemic coincided with the introduction and abuse of antibiotics. I would hate to see this happen again with probiotics. I believe probiotics have a tremendous potential but cannot be used promiscuously for whatever condition they seem to work without customizing the right probiotic for the specific dysbiosis, otherwise we will repeat the same mistake we made with antibiotics. So I think the key elements here, and this is what is called personalized medicine, is to study large numbers of patients, stratify the population because I can't believe that all the individuals are made equal, find the ones that may eventually benefit from a specific intervention because of a specific dysbiosis, customize the probiotics to give to these individuals and objectively evaluate the outcome. That's what I think would be the most logical approach.

Dr. Simmer: I want to ask about the preterm infants. We do know about the microbiome and we know that we well and truly have mucked it up with our frequent and prolonged use of antibiotics. Is there an increased incidence of food allergies or even celiac disease in preterm infants?

Dr. Fasano: I can't answer about celiac disease other than in general terms. Premies have double disadvantage, the one to be most frequently born from C-section and, therefore, to acquire their microbiota from the environment (not preselected to be compatible with or 'good for' his or her own genome) rather than from their mother (that in general has preselected a 'friendly' microbiome for her genome and, therefore, for her baby) during vaginal delivery. The second disadvantage is that the totality of premies are on antibiotic treatment while in NICU, so causing further dysbiosis. It's well known from the literature that premies have more chance to develop allergies over time and, therefore, it is tantalizing to hypothesize that this is due to improper microbiota. Dr. Guandalini, do you know anything about premies and celiac disease?

Dr. Guandalini: No, there is no increased prevalence of premies among patients with celiac disease.

Dr. Simmer: I actually don't think there is any increased incidence in food allergy either.

Dr. Fasano: There are definitely reports showing increased incidence of atopic dermatitis or multiple food allergies in preterm infants and other reports that dispute this increased risk. Therefore, the issue is not quite settled and the jury is still out there.

Dr. Lack: About food allergies, if you are talking about type 1 IgE-mediated food allergies, you are protected if you are born prematurely. As for mucosal non-IgE allergies, there is the perception that this may be higher, so I think it operates differently for both sorts of allergies.

Dr. Papadopolou: Going back to *Bacteroides*, C-section was shown to be associated with almost a complete depletion of *Bacteroides* in the intestinal lumen. Do you know whether C-section is associated with increased prevalence of celiac disease?

Dr. Fasano: Yes, I just told you that there is a group in Canada that showed this increase of celiac disease among kids born by C-section. Again, I don't want to go more into the discussion about *Bacteroides* role but *Bacteroides* produce a substance, a polysaccharide that has a tremendous positive role in facilitating Treg maturation, so it would be tantalizing to hypothesize that if you don't have *Bacteroides* there is a defect in Treg and, therefore, suppression against autoimmune responses. I am aware of several groups studying this phenomenon and await the results of their studies

Dr. Klis: I hadn't seen those data on *Bacteroides* before, and I find them very fascinating. *Bacteroides*, if I remember my bacteriology, is one of the major players in bile acid metabolism since it produces deconjugase which alters the structure of bile salts and affects the turnover of bile acid. Have you done bile acid assays on these kids to see if the lack of *Bacteroides* in their stool correlates with any abnormalities in bile acid metabolism?

Dr. Fasano: We are currently looking at this aspect. As you correctly mentioned, the composition of the microorganism 'village' in the gut, who stays and goes, really depends on so many factors. Again, food is one of them but also the bile acid metabolism and the short chain fatty acid metabolism decide who is going to stay and who goes. So, I believe that *Bacteroides* are definitely susceptible to that kind of change, and we are very much looking into that because that will be a tremendous finding that will explain so many things that we can't explain right now.

Dr. Haschke: One comment and one question. When I was working in the US in 1980, celiac disease didn't exist, and my speculation is that the increasing prevalence in the US has a lot to do with the arrival of Italian GI doctors who provided proper education and had the clinical experience. Could you elaborate a little bit on the so called protective effect of breastfeeding which was shown in the Swedish study? What makes breast milk protective, is it the microbiota which are transferred through breast milk?

Dr. Fasano: Honestly, I think that I can only speculate based on the evidence in the literature. Definitely, the breast milk contains substances that favor some microorganisms and make unfavorable environment for other microorganisms, so it definitely influences the microbiota, no question about that. And again, now we start to see well-designed studies that compare the microbiota of kids on breastfeeding and the ones on formula feeding, and it will be interesting to see what is going to come out of these studies. But I think that it would be reductive to say that it's just simply the effect on the microbiota that makes the difference. I believe that there is also the passive immune response with immunoglobulins present in the breast milk; I really believe that there are molecules that affect antigen trafficking, protection in terms of trophism factors like growth factors that can help to maintain the intestine barrier protected, and again I believe that the other thing that has been overlooked for many years is the capability of the breast milk to train the gut-associated lymphoid tissue

that is forming and is in a very dynamic and yet crucial time in the first 6 months of life to manage environmental triggers that reach the lamina propria. So, I think that goes way beyond the microbiota composition.

Dr. Lima: We made a study in the human milk, and there were different *Lactobacillus* in the women who were on antibiotics during C-section and the babies who were born by C-section, and it's very interesting with regard to *Lactobacillus salivarius* and *Lactobacillus rhamnosus*. We are reprogramming our microbiota at this moment, and I think it's very interesting to continue to study what happens with the reprogramming of our babies in this kind of situation. I think it's very interesting that we also study the microflora in preterm babies and the mothers of the preterm babies; what happens with these, what is the selection in this kind of patients?

Dr. Fasano: I completely agree with your statement. Mother Nature made things the way that they are for a reason, and if we intervene in the process by for example giving antibiotics before a C-section, we definitely affect a very finely regulated process of equilibrium that matured during evolution, no question about that. But I need to make a pledge here, and that probably needs to be stressed more and more. I think that this is the time in which we have an extremely powerful capability to understand the unthinkable, how we interplay with the environment and really put more emphasis on the hygiene hypothesis. However, it would be a travesty if we rush an interventions that are not customized by using pre- and/or probiotics without doing our homework. We will make a disservice, we will definitely pay a price, and with this powerful potential tool that we may have to reprogram and put back in balance – the gut microbiota – can turn against us big time, so I think that people in industry, academia, and legislators need to work in concert to make sure that this is done right. The FDA and the NIH took a major step toward the right direction by requesting specific guidelines on how to use probiotics, what kind of stability they have, their composition and so on and so forth.

Infant Feeding Practices and Subsequent Development of Adipose Tissue

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Abstract

The main aspects of infant feeding that have been studied in humans in association with the subsequent development of adipose tissue include breastfeeding, rapid infancy weight gain, and weaning practices. While observational studies have consistently shown a protective effect of breastfeeding on the development of obesity, these studies may be confounded by unmeasured or unknown factors, as suggested by one study using a sibling design and one study using a randomized breastfeeding promotion intervention design. Observational studies and findings from a limited number of experimental studies suggest that rapid weight gain during infancy may be associated with an increased risk for obesity in childhood and adulthood. The association of weaning practices with later obesity has not been extensively studied, and the preliminary findings are inconsistent. Additional research studies, especially randomized interventions with long-term follow-up, are necessary in order to assess if short nutrition interventions during the critical period of infancy can have long-term benefits on the prevention of obesity.

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The association of infant feeding practices with the development of adipose tissue, in particular excessive adipose tissue or obesity, has been extensively examined [1–4]. This review is not meant to be comprehensive, but rather a critical assessment of selected research findings with an emphasis on adipose tissue measurement methods.

Adipose Tissue in Humans: Measurements and Surrogates

One clinically significant aspect of adipose tissue in humans is that its excessive accumulation, as part of obesity, is associated with unfavorable

health and psychosocial consequences during childhood and adulthood, such as type 2 diabetes, hypertension, dyslipidemia, some cancers, fatty liver disease, polycystic ovary syndrome, osteoarthritis, and social stigmatization [5]. Direct measurement of adipose tissue mass in living humans is not possible; therefore, research and clinical assessments rely on indirect or surrogate measurements of body composition. Body composition indicates that the human body can be partitioned into fat mass (adipose tissue) and lean mass. Lean mass can further be partitioned into bone and lean soft tissues.

Underwater weighing is often considered as the *in vivo* gold standard to measure fat vs. lean mass. Based on Archimedes' principle, body volume can be derived from bodyweight under water and outside water. Using measurement of lung volume and assumption about the density of fat and lean body mass, the proportion of fat mass can be estimated. The advantage of this method is that it is very precise, but it is not widely available or transportable and it requires the subject's complete cooperation to stay still underwater and to measure lung volume, making it difficult to use in children.

In children, dual-energy X-ray absorptiometry (DXA) is often considered as the age-appropriate gold standard for measurement of body composition. Using X-ray properties of differential absorption by calcium, fat, and water (the main component of lean tissue), DXA provides images that can be analyzed to estimate the mass of bone, fat, and lean soft tissues. The advantages of DXA are that it requires only minimal cooperation from the subject and is rapid and reproducible. Its disadvantages are that it cannot easily be transportable to the field for epidemiological studies and that it leads to a small exposure to X-rays. Additionally, there are no undisputable reference data of body composition from DXA available to adjust for the physiological changes during growth and puberty in children and for differences between sexes. Therefore, most studies use an internal reference and compare subjects above and below a specified percentile of fat mass of percent body fat.

A more transportable method of assessment of adipose tissue is the measure of skinfold thickness using a precise caliper and by pinching the skin at specific body locations. The advantages of the method are its low cost and transportability and the existence of reference data [6]. Its disadvantages are that it is very dependent on the observer and therefore requires extensive training and reliability testing to provide reproducible data and that it can be difficult to assess in very obese individuals. Furthermore, skinfold thickness only measures subcutaneous fat and not intra-abdominal or visceral fat, which is thought to be more strongly associated with metabolic complications of obesity.

Several other methods of body composition measurement have been used in children, including total body potassium by whole-body 40K counting, tritium dilution, bioimpedance, and air displacement plethysmography. The limitations of these methods however make them less feasible, less reliable, or less reproducible than the ones listed above.

As can be seen from the above discussion, the direct measurement of adiposity in the clinical setting or epidemiological research setting can be challenging, and surrogates of adiposity based on simple measurement of weight and height are therefore useful in these settings. The most widely used surrogate for measurement of adipose tissue is body mass index (BMI) as defined by the weight in kg divided by the height in meters squared (kg/m^2). BMI is closely correlated with adiposity and performs well at classifying subjects as obese, including children, using DXA as a reference method [7]. BMI is relatively independent of stature, very reproducible, easy to measure with minimal training, and has good reference data to adjust for the physiological differences between ages and sexes [8]. The disadvantage of BMI is that it measures body mass relative to height and not body composition or adipose tissue. Therefore, very muscular individuals can sometimes be misclassified as obese (BMI above the 95th percentile of the reference population for children or 30 for adults), but in the general population of children, this is unusual, and most children classified as obese using BMI truly have excessive adipose tissue [7].

Most studies using a life course approach to obesity epidemiology that examined the association between infant feeding and the subsequent development of adipose tissue are based on BMI or a BMI-based classification of obesity, because the large sample size necessary to demonstrate these relatively small effect sizes makes it difficult to use more direct measures of adiposity. Some studies, however, have used such methods and will be highlighted in this review. Because the risk for negative health outcomes associated with adipose tissue, at least in adults, is not linear, but rather increases rapidly above a threshold close to the definition of obesity [9], a dichotomous outcome of obese vs. non-obese is generally considered as more clinically relevant and preferred as opposed to a continuous measurement of adiposity.

Breastfeeding and Later Obesity

Many observational studies have tested the hypothesis that breastfed infants are at lower risk to become obese than are formula-fed infants. While some early studies were inconsistent [10], most recent studies have consistently shown a negative association between breastfeeding or breastfeeding duration and the risk for later obesity. In one of the most convincing studies, Gillman et al. [11] demonstrated a 'dose-response' effect, in that the longer the children were breastfed, the lower the risk for obesity in adolescence. Interestingly, in a study of more than 4,000 British children aged 9–10 years, Toschke et al. [12] showed no significant association between the duration of breastfeeding and the risk for overweight or obesity as defined using BMI after adjustment for important confounding factors. However, in this same study, they demonstrated a lower risk for having fat mass above the 90th percentile, as measured by DXA, among the children who were breastfed longer.

These results suggest that breastfeeding may be protective against excessive adipose tissue accumulation, even without association with weight status measured by BMI. In a meta-analysis of 28 observational studies published before 2005, Owen et al. [4] found a combined decreased risk for obesity of 13% (95% CI: 11–15%) in formerly breastfed versus formula-fed children and adults, but also described evidence for a publication bias, i.e. a higher chance of positive vs. inconclusive studies to be published.

As consistent as these observational studies might be and as careful as the researchers can be to adjust for confounding factors associated with breastfeeding and obesity later in life, the major limitation of these observational studies is the possibility of unmeasured or unknown confounding factors. In fact, it is difficult to imagine that, even at the same level of education and income, a mother who chooses to breastfeed is not different from a mother who does not choose to breastfeed in her health behaviors and the health behavior of her children in ways that affect the risk for obesity through mechanisms other than breastfeeding. In order to address this inherent limitation of observational studies, Nelson et al. [13] studied pairs of siblings discordant in their breastfeeding history and showed that after taking into account the sibling status, the association between breastfeeding and obesity demonstrated without taking this family relationship into account was no longer present. Another sibling study, however, confirmed an association between breastfeeding and obesity, after taking sibling status into consideration [14].

The best way to demonstrate a causal protective effect of breastfeeding on obesity would be a randomized trial of breastfeeding versus formula feeding. This would not only be difficult to conduct, but unethical, considering all the known benefits of breastfeeding unrelated to obesity. However, it is ethical and feasible to randomize hospitals to optimal vs. usual breastfeeding promotion and support, so that the difference in breastfeeding rate and related outcomes are not linked to confounding factors but due to the randomization to the intervention in ways that strongly suggest causality. Using such a design, Kramer et al. [15] did not show a protective effect of breastfeeding on BMI status at age 6 years, suggesting that the association described in observational studies might be due to unmeasured or unknown confounding factors rather than a true causal effect. However, it is possible that a protective effect of breastfeeding only manifests at a later age or that a more direct measurement of adiposity would provide different results.

Infancy Growth Rate and Later Obesity

Several studies published in the 1970s suggest that a more rapid rate of weight gain in infancy could be associated with a higher risk for obesity later in life, perhaps through a ‘programming’ or ‘imprinting’ effect [16, 17]. In a large US cohort study, we demonstrated a positive association between the

rate of weight gain in the first 4 months of life and obesity at age 7 years, regardless of birthweight [18]. These findings were confirmed in a subsample followed up to young adulthood where rapid weight gain in the first 4 months of life (change in weight for age of 1 standard deviation or more) was associated with obesity, defined using BMI, as well as overweight and over-fat status, defined using a combination of BMI with skinfold thickness measurements [19]. In that study, the population attributable risk for rapid infancy weight gain was estimated at about 30% for obesity 20 years later. Using DXA, Cameron et al. [20] confirmed an association of rapid infancy weight gain with adiposity at age 9 years. Similar results were reported by Ong et al. [21] in 10-year-old girls also using DXA to measure adiposity. In a meta-analysis based on subject level data from 9 studies, Druet et al. [22] reported a combined 93% increased risk for childhood obesity and a combined 22% increased risk for adult obesity for each one standard deviation increase in weight-for-age in the first year of life.

As for the association of breastfeeding with later obesity, the association of rapid infancy weight gain with later obesity in observational studies could be explained by unmeasured or unknown confounding factors. To address this limitation and better adjust for family-level confounding factors, we analyzed data from siblings discordant in their infancy weight gain, and found evidence that the association of rapid infancy weight gain with obesity at age 7 years is not due to family level confounding factors [23].

As part of a series of experimental studies of cardiovascular risk factors in children who were born premature and fed different types of infant formula for the first few weeks of life, Singhal et al. [24] showed that the subjects assigned to a formula that led to an improved weight gain during these first few weeks of life showed signs of leptin resistance, an endocrine condition associated with obesity but not with a significantly increased weight status. In another experimental study of subjects born at full term and randomly assigned to a formula with lower or higher protein concentration, Koletzko et al. [25] showed an increased weight gain in the first year of life that was followed by an increased BMI at age 2 years. Taken together, these studies suggest that there might be a causal link between rapid infancy weight gain and later obesity and could lead to new approaches to obesity prevention during short critical periods of life.

Weaning Practices and Later Obesity

The American Academy of Pediatrics recommends feeding exclusively breast milk, or formula for non-breastfed infants, for a minimum of 4, but preferably 6 months [26]. However, the practice of introducing other foods, especially cereals, before 6 and even 3 months is frequent in the US [27], and has been hypothesized to contribute to excessive infancy weight gain and subsequent development of excessive adipose tissue. A recent study of

42-year-old adults showed a decreased risk for overweight or obesity by 10% (95% CI: 2–19%) for each younger month of age at introduction of vegetables, by 7% (95% CI: 0–13%) for meat, and by 8% (95% CI: 2–14%) for firm foods [28]. No statistically significant associations were observed for age at introduction of spoon-feeding or eggs. Burdette et al. [29], however, did not show a statistically significant association between adhering to the American Academy of Pediatrics guidelines on age at introduction of complementary foods and high levels of adiposity measured by DXA in 5-year-old children.

Conclusions and Research Needs

Breastfeeding is associated with a lower risk for obesity later in life in many observational studies, but not in the only published randomized study. Rapid infancy weight gain is associated with subsequent obesity in many observational studies, but the limited experimental studies are inconsistent. Evidence for an association of timing of introduction of complementary feeding with later obesity is inconclusive.

Based on this brief review, the following research areas can be identified. Although there is no need for additional observational studies of the association between breastfeeding or rapid infancy weight gain with later obesity, experimental studies for these two exposures with long-term follow-up are critical and should be research priorities. Additional observational studies, meta-analyses, and experimental studies investigating early introduction of complementary food as a risk factor for subsequent development of adipose tissue are necessary. Although measures of obesity based on BMI are adequate, due to some contradictory results, more direct measurements of adiposity are useful when possible in the research setting. The study of the association of infant feeding practices with the subsequent development of adipose tissue may lead to novel approaches to obesity prevention targeting short critical periods with long-term benefits.

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Discussion

Dr. Siega-Riz: I have an observation from one of the studies that we're about ready to publish. That study actually showed that children fed lower nutrient formula ended up having a lower likelihood of developing obesity later in life. It struck me that in fact the breastfed babies lose weight in the first week of life. That's a very normal and natural thing for them to do. So, in fact that's sort of mimicking what is happening, and I think what you are saying is fetal program is contributing to something, but there must be some programming going on in that first week of life that is very critical.

Dr. Stettler: Yes, that was the point of that study that was presented at the Obesity Society Meeting, where they showed a very clear weight loss on the modified formula in the first week of life. They had a control group (obviously not randomized) of breastfed infants who also lost weight. The infants on regular infant formula hardly lost any weight. Those on the experimental formula mimicked more closely what breastfed infants did.

When we first found in an observational study the importance of the first weeks of life [1], I initially doubted that it would be reproduced and that it might have been a chance finding. But now I think there is something going on, and we need to think about it. This is the period when for the first time, human beings have to regulate how much they are eating. This is really the time when they are learning what hunger is, what the cues are, what is happening when they eat (they are less hungry), how much they need to eat, and so forth. It's also a time, as you know, when there is a lot of the wiring in the brain that is happening, so if you think in these terms, it would make sense that the learning experience in this very early stage could have a long-term impact. It has actually been demonstrated in animal models where they could look at the brain and the influence of feeding in this very short period of time [2]. So, I think that that could be a really interesting new approach.

What I didn't mention is that obviously these data have to be seen in the context of risks and benefits. For breastfeeding promotion, there is really very little risk associated with it, and there are a lot of other benefits, so in terms of clinical practice or public health it's easy to recommend breastfeeding, even if the evidence for obesity prevention is not very strong. When it comes to restricting weight gain in infants, it's a different question. There is a lot of evidence that decreasing rate of weight gain can be associated with negative effect on infection, on the brain, and on statural growth. So obviously, those findings are not directly translatable to clinical practice or to public health, but they are interesting when we try to think about programming and early prevention.

Dr. Siega-Riz: In our study, we followed pregnant women all the way through the first year of postpartum, and then we followed the kids up to 3 years of age – very similar to Matt Gillman study. In our population in which 60% of the women are breastfeeding, the thing that seems to be associated with increased waist status at age 3 is introduction of complementary foods before 4 months of age.

Dr. Simmer: In your presentation, you didn't mention air displacement plethysmography or even ultrasound.

Dr. Stettler: I was limited with time and just wanted to show the methods that were included in the studies that I presented. There are several other ways to measure body composition; air displacement plethysmography is one of them; it uses the same principle as underwater weighting, and has similar disadvantages, in that it requires the collaboration of the subject in terms of breathing. Our experience with this method in children and infants is not very good. So that's one another reason why I haven't presented it. The other way that is really popular, but I also don't think is very good, is bioimpedance. It measures how much electricity goes through the body and, based on

that, derives the percent body fat. These two methods are actually OK to show correlations between methods in percent body fat, but when you look at the individual between-method differences, they are pretty large.

Dr. Harding: I am glad you raised the issue of risk-benefit because I think we do have to be worried about what we are preventing here. If we go back to the work of Lucas and Singhal, preterm babies randomized to increased nutrition grew faster and had better developmental outcomes [3]. However, cardiovascular risk and obesity were related to weight gain [4], which raises the question of causality. Rapid postnatal weight gain may reflect recovery from a prenatal restriction, and in animal studies it is the prenatal restriction that results in the long-term disease risk, independently of postnatal weight gain. So, are there any human studies where people have looked carefully and sequentially at prenatal growth? You would need to do repeated ultrasound measurements of growth, and then relate that to postnatal growth and to the long-term outcomes.

Dr. Stettler: Those are all good points, and I think the one thing that I can add to your comment is that unlike in animals, infants who are born with intrauterine growth retardation are not at higher risk of obesity. They are at higher risk for cardiovascular disease but clearly not obesity, which is not the case in rats. If you restrict fetal growth in rats, they will catch up, and then they will become obese and diabetic. The question whether the rapid weight gain is related to intrauterine growth retardation is the one we try to address when we stratify children by birthweight, and we still see an association between weight gain and obesity in each of the 5 birthweight categories.

Dr. Harding: I think we do then run into the problem of birthweight versus fetal growth, and again it doesn't really address the question of causality.

Dr. Öhlund: I have a question regarding obese women. Obese women can breastfeed less well than normal-weight women. How do you think that affects the children?

Dr. Stettler: One of the strongest predictors of childhood obesity that I didn't talk about is maternal obesity, and many things could explain this association. Obese women are more likely to have diabetes during pregnancy, which is a strong risk factor. They are more likely to have bigger babies at birth, which has also been shown to be a risk factor. They are less likely to breastfeed, which could also be a risk factor. And obviously, the genes are transmitted and the environment is common. So there are a lot of reasons for the association between obesity in the mother and obesity in the child. The question is what are the intervention implications? One of the things that are most exciting is to address weight gain during pregnancy, which is strongly associated with obesity in the offspring. When they become pregnant, many women want to try to do everything right for their baby, so it may be a good opportunity, a good timing, for a preventive intervention for themselves and for the baby.

Dr. Klish: Just a couple of comments about both growth and the measurement of adiposity. In the first week of life, weight loss is usually due to a loss of total body water, but this can happen simultaneously with an increasing lean body mass. So, measurements in that first week depend on how growth is being assessed. I also have a concern about DXA becoming the gold standard of adiposity. When we studied all these methods in the 1980s, the standard error of the estimate for DXA was somewhere in the range of 2 kg of fat. At a young age, the standard error for BMI is about the same. BMI very poorly correlates between adiposity and lean body mass in the normal range, but it correlates better above a score of 30. I think the same thing is true of DXA. You probably pointed out why when you showed the halo effect of DXA. We know from the few studies of total body analysis of the human body that only about half of the adipose tissue in an adult human is in the subcutaneous space. The rest is deep within the body, as marbling of muscle, perinephric fat, omental fat,

things of that nature. So, we have to always be very careful as to how we interpret DXA.

Dr. Stettler: I agree, DXA has its limitations, I think it's becoming the gold standard for practical reasons because there is really no better practical alternative. The funny thing about DXA is that reports give a very precise number for fat mass, at the 10th of a gram level, so many people think that DXA is precise to the 10th of the gram. This is really misleading, and one has to remember not to take into account those last digits, because the method is not that precise. Also, I am not too concerned when people use BMI, especially if they are using it as a dichotomous variable, because the vast majority of kids who are above the 95th percentile for BMI really are obese, they have too much fat around.

Dr. Chittal: If we have weight loss in full-term babies, acceptable weight loss, why are we so keen on feeding preterm IUGR babies very aggressively in the first week, aren't we programming for a future obesity by doing so?

Dr. Stettler: It's a good question. The situation is very different between full-term infants, preterm infants and infants with intrauterine growth retardation. For most preemies, we are not able to even keep them on their growth curves, they all lose weight or gain insufficient weight. So, I don't think overfeeding them is a concern; it's really trying to keep them at the expected level of weight gain, and this has been shown to be associated with neurodevelopmental benefit. It's a question of priority, do you want to have a preterm baby who has a better neurocognitive development and maybe has an increased risk of obesity, or the opposite? But I am not even sure about the risk of obesity in these situations, because excessive weight gain is not an option, we are just trying to get them to grow at a healthy rate. It is really when the baby is in the excess weight gain range that we are concerned about obesity, not in the normal weight gain or in the low weight gain range.

Dr. Lack: I wondered whether there are any data relating to protein intake, on the quality of protein, vegetable versus animal and, say, soy feeds versus cow's milk-based feeds, and in older children vegetarian versus normal diets.

Dr. Stettler: I am sure there are. I really don't know much about that topic. Berthold Koletzko in Germany looked at protein in detail, I think; infant formula manufacturers have also looked at that.

Dr. Klassen: We actually have performed some studies where we could show results going in the same direction as Berthold Koletzko's study.

Dr. Stettler: He was asking about the quality of the protein, so is there a difference for example between soy protein and cow's milk protein?

Dr. Klassen: To my knowledge, what has been studied is infant formula based on cow's milk protein. A low-protein, high-quality infant formula at the lowest limit of current legislation (1.8 g per 100 kcal) can basically be achieved by using a whey-predominant formula since this will allow to supply sufficient amounts of the limiting amino acids. Randomized controlled studies have been performed demonstrating that these formulas result in appropriate growth of the child and also an appropriate amino acid profile. I am not aware of any data for soy, so for the early period of life it's only cow's milk protein I am afraid.

Dr. Van Goudoever: I have a remark and a question. The goal for premature infants from a feeding perspective is not to let them drop through the standard curves basically. So it's not too much about weight gain, it's more about preventing weight loss, severe weight loss. My question is related to the measurement of fat on ultrasound because in my opinion it's not only the total amount of fat mass but also the position where it is. What are your ideas about using ultrasound to measure peritoneal fat?

Dr. Stettler: This would be really wonderful, I haven't seen good reference data, and I think that's the limitation. You may be aware that this is happening now, and

this would be a great alternative. Intra-abdominal fat so far has been measured with CT scan and MRI that have made it difficult. We need data on ultrasounds and good reference in kids, which is actually really difficult to have now considering that most countries have an obesity problem, but if there is a good reference database, that may be a good way to address it.

Dr. Haschke: Dr. Yajnik has published MRI data from India and UK on distribution of visceral fat versus subcutaneous fat of newborns. Indian newborns have lower birth-weight but already more visceral fat.

Dr. Stettler: This is where ultrasound is going to be very helpful. What I said about intrauterine growth retardation in human not being at risk for obesity is true, but it is a risk for those who do become obese to have a more central fat distribution. This is where the metabolic aspects are becoming more important.

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Early Life Nutrition and Bone Development in Children

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Abstract

Fetal and early life may be a critical period for the development and/or programming of metabolic systems, including the skeleton. There are increasing human data from cohort studies on the association between early life nutrition and bone development in children. Breastfed children initially have lower bone mass than bottle-fed children, but longer-term studies suggest that they have higher bone mass (size adjusted) by age 8 years, especially in children born at term. By the time of peak bone mass, both preterm and term children have higher bone mass indicating a different bone accrual trajectory curve. These children also have lower fracture risk. Diet in utero has also been associated with subsequent bone mass from ages 6 to 16 years (but not fracture). Positive associations include milk, phosphorus, magnesium, potassium, protein, folate, calcium and vitamin D, while fat intake is negative. Smoking also interferes with bone mineralization possibly due to impaired placental function, but this deleterious effect on bone mass appears to diminish over time. All of these associations are statistically significant and independent of important confounders and later environmental exposures, suggesting that osteoporosis prevention programs need to start very early in the life cycle.

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Introduction

Fractures are a major public health problem in males as well as females [1]. Bone density is one of the major predictors of these osteoporotic fractures in both the elderly [2] and children [3], and is the result of the amount of bone gained in early life (i.e. peak bone mass) and subsequent bone loss [4]. Physical activity and, to a lesser extent, diet (particularly calcium intake) during adolescence and early adulthood have been implicated as determinants of peak bone mass [5, 6]. However, the vast majority of adult bone mass

is attained before the age of 14 years [7]. In recent years, evidence has accumulated in support of the Barker hypothesis [8] for bone development for in utero diet and breastfeeding among other factors. The aim of the review is to summarize this literature relating to later effects of in utero and early life exposures for in utero diet, breastfeeding and smoking (as this may impair nutrition).

Diet in Pregnancy

Nutritional influences on childhood bone development may begin in utero, and because of in utero programming, such influences may affect both early skeletal development and the acquisition of bone mass throughout childhood. Studies examining this are few but there seems to be a consistency of results. In an early exploratory study from my group, maternal dietary intake of magnesium, phosphorus, potassium and protein during the third trimester of pregnancy was positively associated, and maternal fat intake negatively associated, with bone density in their children at age 8 [9]. In an English cohort [10] reporting associations between maternal diet at 32 weeks gestation and bone mineral content (BMC) and bone mineral density (BMD) at age 9 years, maternal magnesium intake was positively associated with total body BMC and BMD, but this did not persist when they adjusted for height. Furthermore, maternal intake of potassium was positively associated with spinal BMC and BMD, but this decreased after adjustment for weight. Both of these suggested an effect mediated through body size which differs from the Tasmanian data where the effect was independent of body size. Maternal folate intake was positively associated with spinal BMC adjusted for BA in the English cohort after adjusting for both weight and height of the children. The effect sizes in this study were smaller than those observed in the initial Tasmanian study [9]. In an Indian study [11], 6-year-old children of mothers who had a higher frequency of intake of calcium-rich foods during pregnancy (milk, milk products, pulses, nonvegetarian foods, green leafy vegetables, fruit) had higher total and spine BMC and BMD, and children of mothers with higher folate status at 28 weeks' gestation had higher total and spine BMD, independent of parental size and DXA measurements. These children were smaller and lighter than norms, suggesting they were less well nourished, and the authors state that the results may reflect protein intake as well as micronutrients.

To date, studies have followed children up to age 6–9 years but not through puberty, so it is unclear if these associations are short- or long-term. Our recent report in 16-year-old children provides evidence in a well-nourished population that milk, fat and magnesium intake during pregnancy can independently influence bone mass for at least 16 years [12], and is thus likely to impact on peak bone mass. These results were similar (but smaller in magnitude) to

Table 1. Dietary factors and their association with spinal bone mass in Tasmanian children

Dietary factor	Age	
	8 years	16 years
Calcium	no association	no association
Phosphorus	positive	positive
Potassium	positive	no association
Magnesium	positive	positive
Fat	negative	negative

the results at age 8 years (table 1). Given the very small amount of bone laid down during pregnancy, this association most likely reflects early programming of later bone responses. Maternal magnesium density was positively associated with BMD at the femoral neck of 16-year-old adolescents, which is partially consistent with our previous report where it had strong associations in adjusted analysis that did not persist after adjustment for other dietary factors and the report of Tobias et al. [10]. Although the precise mechanism of maternal magnesium intake affecting bone health of offspring is currently unclear, it might be involved in changes of fetal calcium homeostasis and calciotropic hormones. Because magnesium is known to compete with calcium for binding to the calcium-sensing receptor, leading to a reduction in parathyroid hormone secretion, increased maternal magnesium intake has the potential to lower maternal serum calcium concentration. Another possible explanation is that magnesium intake is also positively associated with birth weight. However, adjustment for body size and/or birth weight did not alter our findings. Maternal milk intake density during pregnancy was positively associated with BMD of 16-year-old adolescents in this study, which is again consistent with our previous report [9]. Milk contains many potentially growth-promoting factors and is associated with higher birthweight for gestational age. Furthermore, a study in a group of pregnant African-American adolescents found that nutrition was significantly related to fetal femur growth during pregnancy, such that dairy intakes of 2 servings per day were associated with lower fetal bone development than were greater intakes of dairy [13]. Phosphorus density was associated with BMD of 8-year-old children but not of 16-year-olds, whereas calcium density was not associated with bone health in either 8- or 16-year-old children (possibly due to their high average intake). Tobias et al. [10] found that maternal phosphorus intake was positively related to total body BMC and BMD of 9-year-old children, but there are no other reports in adolescent children. It may be that phosphorus has a short-term effect rather than a long-term effect. In all of these studies apart

from the original Tasmanian report, the proportion of variance explained by diet is small being in the order of 1–2%, and there has been no association with fracture to date. This may suggest a trivial effect, but there is substantial error with dietary questionnaires which is likely to weaken associations, and stronger results may be achieved using more comprehensive dietary assessment such as weighed food records.

Randomized controlled trials examining childhood bone outcomes from supplementation interventions in pregnancy are lacking. However, maternal vitamin D supplementation in pregnancy resulted in lower bone-specific alkaline phosphatase levels and smaller fontanelle size (suggesting improved skull ossification) [14] in infants, and in lower cord serum alkaline phosphatase [15] and greater crown-heel length [15] in neonates. Zinc supplementation in pregnancy in a poor area in a developing country resulted in increased fetal femur diaphysis length [16]. In a retrospective cohort study, maternal use of vitamin D supplements was associated with increased BMD at the distal radius and femoral neck, though not lumbar spine [17], and maternal vitamin D status during pregnancy also predicts bone mass in their children at age 9 years [18]. Though limited, these data provide support for further research into nutritional interventions in pregnancy.

Breastfeeding

There are limited data for mode of feeding in early postnatal life. There is controversy about short-term effects, with most studies showing a deficit in bone mass in breast milk versus formula-fed infants and one showing no effect with evidence suggestive of a catch-up phase by 2 years in one of these cohorts [reviewed in 19]. In a long-term study of preterm infants, those supplemented with banked donor breast milk for the first 4 weeks of life (regardless of type of infant feeding), had improved bone mineralization at the radius up to age 5 years [20] but this did not persist at age 20 years, although there was an association between the percentage of breast milk in the diet and whole-body BMD at age 20 years [21]. These studies have been restricted to preterm infants and cannot be generalized to term infants as unsupplemented breast milk may not fully meet the mineralization requirements of preterm infants [22]. There are less data in healthy children. Harvey et al. [23] reported no association at age 4 years, and we have previously reported a beneficial association of breastfeeding for both bone mass [24] and fractures [25] up to age 8 years in children, especially in those born at term. Other observational studies with bone measures at younger ages did not demonstrate associations between breastfeeding and bone density [19]. However, in a retrospective study, premenopausal women who had been breastfed for more than 3 months had greater cortical thickness at the radius and a trend towards greater cortical area and cortical BMC at the radius, but not at other sites [26].

Table 2. Associations between breastfeeding and bone mass in Tasmanian children

Study factor	Spine	Hip	Total body	Fractures RR
Breastfeeding (yes vs. no)				
Age 8 (refs.)	+1.03%	+1.02%	+1.02%	0.40
Age 16 [24]	+3.42%	+2.84%	+2.85%	0.69

Bold denotes statistical significance.

Bone density tracks strongly from age 8 to 16 years, but a minority of children track deviate from tracking in either direction, i.e. up or down [27]. Children who were breastfed had a different trajectory to non-breastfed children being twice as likely to deviate upwards in terms of their bone accrual trajectory and half as likely to deviate downwards [27]. At age 16 years, the magnitude of effect from breastfeeding is greater than at age 8 (table 2), and there was also a reduction in incident fracture risk between age 8 years and age 16 years (table 2). This supports the concept of breastfeeding programming bone accrual (independent of linear growth). Breastfeeding is related to socioeconomic status; thus, it is possible that any association may be mediated by other factors. However, in our cohort, the associations persisted after adjustment for lifestyle and socioeconomic factors (apart from maternal education) and appear dependent on the duration of breastfeeding, frequency of night feeding and the percentage of breast milk in the diet suggesting a biologic association. Indeed, intention to breastfeed at birth was less strongly associated with bone mass than actual breastfeeding. Lastly, the reduction in fracture risk at both age 8 years and during puberty was largely mediated by higher bone mass, which is also most consistent with a biological association.

It is important to note that the infants selected to participate in the original Tasmanian study were not a random, representative sample of children from Southern Tasmania. Six ‘selection factors’ that increased the risk of cot death were used to identify children. These were: gender (males), birthweight (low $\leq 2,500$ g), maternal age, month of birth, duration of second stage labor and intention to breastfeed. Twenty percent of children born each year from 1988 to 1996 were selected. This created a sample with some biases. They were more likely to have mothers who smoked during pregnancy and less likely to have been breastfed. This increased study power, and adjustment for study factors did not change results, suggesting they are generalizable to other western populations of well-nourished children using the guidelines suggested by Miettinen [28].

Smoking in utero

Smoking during pregnancy has also been associated with lower bone mass but not fracture in many studies. One study suggested this was dependent on term of gestation and that the association disappeared after adjustment for placental weight [29]. Placental weight has been associated with bone measures in Indian children [11], suggesting smoking interferes with bone development through intrauterine placental function which is different to how it is thought to influence bone mass in adults. Interestingly, in the Tasmanian cohort, the effect of smoking was no longer evident at age 16 years, with virtually no difference (even though the children were shorter and weighed less), suggesting the bone effect is transient and recovers [Jones, unpubl. data].

Conclusion

There is increasing evidence that early life nutrition has independent associations with subsequent bone mass and fracture up to age 20 years in prospective studies. Only a small amount of bone is laid down during pregnancy, so it seems unlikely that these factors influence this to any major extent. Thus, the data as a whole are more strongly in support of programming, suggesting that osteoporosis prevention should start very early in the life cycle.

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Discussion

Dr. Klish: I would like to make a comment about DEXA measurements with regard to bone density. DEXA is based on the absorption of X-ray by the tissues it passes through. If you put fat in front of the bone you are going to absorb some X-ray before it gets to bone. That will give a different signal than in somebody who is lean. I know

there have been formulas developed to try to correct this problem and would like to know if you tried to correct for this.

Dr. Jones: There are a number of ways to deal with that issue. It's particularly a problem for overweight children and BMC, but bone mineral density is much less affected by body fat. There is, however, a correlation between body fat and bone density which probably is a true association in terms of cause and effect. I prefer weight adjusted for height, which gives a very good measure of body fat in children and works better than BMI because BMI is age dependent. You can also measure abdominal fat quite well with DXA by positioning cursors. In children, this is a reasonable approach. In older adults it's more difficult because you get fat within muscle as you get older.

Dr. Agarwal: We measure bone growth, bone mass and fracture rates at the age of 12, 16 and 20 years. What about the role of sexual development in these children? The sexual development in a child of 8 years will differ from that of a 16- and a 20-year-old.

Dr. Jones: That's an excellent question and one that we have looked at extensively. We found pretty similar correlations between age, height, weight and/or Tanner stage and bone mass. So, you can adjust for Tanner stage or you can adjust for age, height and weight.

Dr. Stathatos: Have vitamin D levels been measured during the study?

Dr. Jones: In our population, roughly 50% of the population is vitamin D deficient with a level below 50 nm. The only group that isn't is the 8-year-olds, and there are roughly 10% of them below 50 nm. Vitamin D is related to bone mass and bone turnover markers in our children, and we have been trying to get money to do a trial from our government, so what we did was a meta-analysis. This shows that vitamin D supplementation works for bone mass if your vitamin D level is below 35 nm, but above that it does nothing. So I am a bit concerned about the apparent epidemic of vitamin D deficiency at present. There is a lot of reverse causation in terms of vitamin D levels and diseases, e.g. if you have juvenile arthritis, your vitamin D will be lower because you spend less time outside. Further, if you look at the relationships, they are not linear, they tend to be logarithmic, so there is more to be gained between 10 and 25 (US 4 and 10) than there is between 25 and 50, than there is between 50 and 100. So you are getting progressively less benefit the higher you go up the scale. So, even in the bone area which has been very well studied it's still very controversial in adults as to what level you should treat. Personally, I think treating 25–30 is mandated, I think there is good enough evidence for that, and I suppose I am most comfortable getting everyone above 50.

Dr. Puri: Our studies [1] have shown that diet really had no relationship with the vitamin D status, that it was mainly lifestyle factors. In a country like India, it is mainly exposure to sunlight. These factors are very important, especially when we are talking about school children. Were these factors built into the study to see the effect on BMD?

Dr. Jones: Yes, and our results are essentially the same as yours. There was no association between vitamin D assessed by the diet and vitamin D levels, so the children mainly get it from outdoor physical activity. That's true at both 8 and 16 years; however, by age 16 the outdoor activities become trivial, and what we need is UV-emitting computers because that's the only way they are going to get their vitamin D. We have done intervention trials with big doses, and we can find that 300,000 U every 6 months prevents everyone dropping below 50 nm.

Dr. Guandalini: Somehow related to vitamin D, I have a question related to dietetic needs of calcium later in life. We have recommendations in western societies of very high intake of calcium. The recommended intake has in fact been recently revised up to 1,200 and even 1,600 mg per day during adolescence. Now, the general

assumption is that such intakes can only be fulfilled by drinking large amounts of milk. So my question is purely conceptual. Cow's milk is for the young of the cows, it's not intended by mother nature for human consumption, and cows have only been domesticated as recently as 10,000 years ago. Hence, how could we be dependent on milk? Is it possible that there are no other sources of calcium? Are green leaves not enough to accommodate for our calcium need? If not, then is it conceivable that our need has been a bit overestimated?

Dr. Jones: I gave a debate in Sydney a month ago on why not to give calcium supplementation. With the meta-analysis in children, we showed that moving average intake from 700 mg a day (which is average in the population) to 1,200 did nothing. When you go back and look at the balance studies, they put people on a very high fixed intake of 1,200–1,500 mg a day. Intake is the main determinant of calcium balance. More recent balance studies have shown that most people can balance their calcium around to 600–700 mg a day. So, I personally think the IDRs are incredibly high and inflated. It is hard to get enough from non-dairy sources. You can get it from can fish, but it has to be fish with the bones in the can so sardines and salmon but not tuna, you can get it from sesame and figs as well. But the body has the ability to vary calcium absorption from 10 to 50% of the diet, so we can actually vary it a great deal and you have got to get down extremely low before you get hypocalcemia or osteomalacia from calcium deficiency probably in the order of 200–300 mg a day.

Dr. Guandalini: So, our huge dependence on milk from another mammal species seems awkward, and I take it you would agree that these recommendations are on the excessive side.

Dr. Van Goudoever: A technological question again. We were talking about the DXA scans, and there are new machines now on speed of sound, and scattering of ultrasound measuring bone density. What are your thoughts about that?

Dr. Jones: I will send you my paper on ultrasound in fractures. In these children, I measured bone in multiple ways. Calcaneal ultrasound was a good predictor of fractures in teenagers. However, it doesn't work in young children because the heels largely have a couple of bits of bone floating in fibrocartilage. I think the most promising is high-resolution CT, which is low radiation and can image down to 80 nm, and that can actually give you a picture of bone structure in vivo.

Dr. Fasano: Another philosophical question related to the main topic you covered concerns the biology of the bone as a result of the balance between osteoclasts that destroy your bones and osteoblasts that build them up. Of course, osteoblasts need vitamin D, but physical activity is a tremendously important aspect of the story. Are there any data in the literature explaining how the health of bones of our kids is changing with the change in the habit of their physical activity?

Dr. Jones: Good data from the US were published in *JAMA* a couple of years ago showing that forearm fracture rates have been increasing in the US over the last 40 years, and there are probably two reasons for that. One is children are getting less active, and secondly they are getting fatter, and the bigger you are the harder you fall. Coca Cola and television watching also increase fracture risk. So, the worry is hip fractures in the elderly might be going down because people are getting more obese, but fractures during puberty are going up.

Dr. Stathatos: Any comment on vitamin K to help the synthesis of connective tissue in bones?

Dr. Jones: Vitamin K is important for osteocalcin, which is an enzyme produced by osteocytes. In bone, there is about 100 million of these cells, but we are not totally sure what they do. In children, there are no data that I am aware of for vitamin K and bone. In adults, meta-analyses show that vitamin K supplements decrease fracture risk, but this has mainly been taken up in Japan.

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IGF-I Signaling and Effects on Longevity

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Abstract

Insulin and insulin-like signaling regulate survival and lifespan in a variety of animal species, from nematodes and flies to higher vertebrates and mammals. Recently, it was shown that brain IGF-I receptor and brain IRS2 control mammalian lifespan, and that this occurs through neuroendocrine mechanisms, control of energy metabolism and modified stress resistance. Furthermore, it was demonstrated that insulin receptor substrate molecules are implicated downstream of insulin and IGF receptors in the extension of lifespan. We showed recently that early postnatal diet plays a significant role in the development of the somatotrophic axis, and that part of the neuroendocrine plasticity of growth hormone secretion depends on postnatal nutrition. We also showed that the prevalence of cardiovascular and metabolic pathologies varied with the development of somatotrophic function. Neuroendocrine pathways are also prime targets for pharmacological treatments, and administration of rapamycin to adult mice has indeed recently been reported to prolong lifespan in mice. With respect to human aging, new studies identified several genes of the somatotrophic axis as longevity determinants, and a recent study shows that variants of FOXO3A, downstream signaling molecule in the insulin/IGF pathway, are associated with extreme longevity in humans. Finally, several functional mutations of the human IGF-IR have been discovered in centenarians.

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Longevity and Aging

From simple multicellular animals to higher vertebrates and mammals, organisms age as they live. As a universal and physiological process, aging occurs simultaneously and in coordinated fashion in all cells and tissues of the body, sparing only the germline. At the cellular and organ level, aging can be described as a progressive functional decline. These changes start early in life, most often as a loss of functional redundancy and without direct consequences for the individual at the beginning. Recent works suggest that the overall

process of biological aging is coordinated by the combined action of genes. On the molecular level, aging can be described as the sum of damages affecting all components of the cells, including DNA and other nuclear structures. Molecular changes due to aging are extremely diverse, although the underlying causes may be less complex. Aging is not a pathologic process and also clearly distinct from disease. However, numerous diseases are associated with age, and the many interactions between aging and disease considerably increase the complexity of aging phenotypes. A large number of premature or accelerated aging syndromes have been identified, and many have now been linked to mutations in genes participating in DNA maintenance and repair. These syndromes mimic physiological aging processes, generally in a segmental or partial manner. As in other domains, research into aging has been largely descriptive for many years, but more recently, experimental strategies to unravel genetics and molecular mechanisms of aging yielded tremendous progress. Much of this was initiated by studies using simple organisms, sufficiently small and short-lived so that screening for mutants with increased lifespan was possible. In the early 1990s, genes regulating survival and lifespan were identified in *Caenorhabditis elegans*, and their roles confirmed by mutagenesis in *Drosophila* [1, 2]. The majority of these longevity genes interact with each other, and it was shown that they are part of a major signaling pathway. The corresponding homologous pathways in vertebrate species and mammals were then identified as the cascades that regulate growth and metabolism, including the widely distributed receptors for insulin and IGF, and their respective substrates, the insulin receptor substrate (IRS) family of intracellular signal transducers. In the following years, results were swiftly transposed from nematodes to mammals, and today, numerous mouse strains with mutant insulin and IGF genes have been extensively studied with respect to aging and longevity.

Growth Hormone, IGF and Insulin Control Longevity in the Mouse

Strong increase in lifespan has been observed in mice with loss-of-function mutations of genes acting upstream of IGF receptor (IGF-IR), namely those implicated directly or indirectly in the neuroendocrine regulation of the somatotrophic hormone (STH, or growth hormone – GH). This is the case for Prop1 and Pit-1, potent transcription factors regulating differentiation of cell lineages in the developing anterior pituitary. Both genes control the somatotrope cell population and thereby ultimately the production of GH in the adult pituitary. GH in turn stimulates the secretion of IGF-I, the cognate ligand of IGF-IR, during postnatal life and also in the adult organism. Inactivation of Prop1 and Pit-1 engenders a pan-hypopituitarism marked by drastic GH decrease. Very low GH in these mutants leads to up to 50% increase in mean lifespan, but also to radical changes in metabolism, growth and fertility [3, 4].

This rather broad phenotype can be attributed to the high degree of integration of neuroendocrine regulations that occurs in the hypothalamic-pituitary complex. Spontaneous mutation of GHRHR also significantly increases survival and lifespan [5]. Similarly, targeted mutations of the GH receptor (GHR), insulin receptor and IGF-IR were shown to increase the lifespan of mice [6–9]. Downstream of these cell surface receptors, constitutive inactivation of p66Shc and IRS1, and the brain-specific knockout of IRS2 also increased lifespan [10–12]. This and other works confirmed that numerous components of the somatotrophic hormone axis are involved in lifespan regulation. In addition to the above, it was also shown that somatotrophic signals determine the resistance to oxidative stresses in several of these mutants. Research into aging in mammalian species has since focused on endocrine and neuroendocrine regulations of the somatotrophic function.

Summarizing the above, three aspects are fundamental: (1) the implication of insulin and IGF signaling in lifespan regulation is strongly conserved during evolution, indicating that this trait is generally useful and important for survival; (2) in mammals, the principal regulator of lifespan is a hormonal signaling pathway that controls several vital processes; (3) the entire somatotrophic hormone axis, which is the main regulator synchronizing growth and development in vertebrates, is involved in lifespan regulation.

Neuroendocrine Plasticity and Lifespan

Hormonal circuits and regulatory feedback allow individuals to adapt to changing environmental conditions through phenotypic plasticity. We showed recently in the mouse that it is sufficient to reduce the circulating levels of GH and IGF-I to postpone age-related mortality and increase lifespan [13]. For that we used the brain-specific heterozygous inactivation of IGF-IR, a conditional mutation that leads to a defect in somatotrophic development shortly after birth. (In contrast, complete inactivation of IGF-IR specifically in the brain leads to microcephaly, profound retardation of pre- and postnatal development, and strong behavioral deficits.) The heterozygous knockout of brain IGF-IR, which produces a partial insensitivity of the brain for IGF-I signals, results primarily in a selective developmental defect of the somatotrophic axis. In that mutant, the development of pituitary somatotropes is retarded; they secrete only a fraction of normal amounts of GH, and mutants show chronically low circulating IGF-I levels [13]. This occurs from shortly after birth onwards and causes a growth deficit phenotype. As adults, these mutants are slightly, but significantly smaller than control mice and their somatotrophic tone is low. The reason for this GH-deficient phenotype resides in the hypothalamus, where low IGF stimulation inhibits the expression of Pit1, that later on leads to a diminished secretion of GHRH at the level of the median eminence. The resulting somatotrophic insufficiency is characterized by strongly

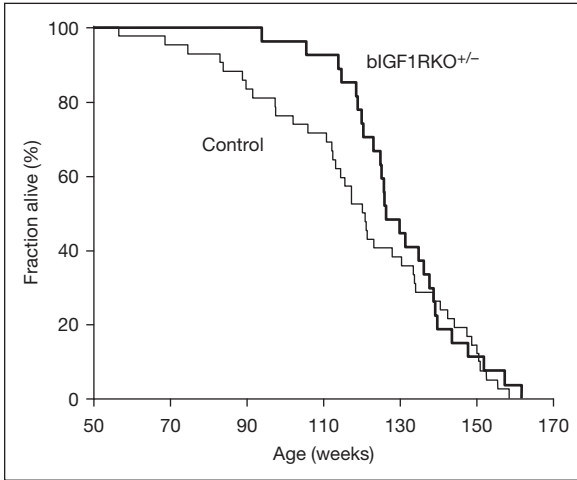


Fig. 1. Extended mean lifespan in mice with heterozygous knockout of the IGF-IR receptor in the brain. Variability of lifespan is much reduced among mutant mice, compared to littermate controls. From Kappeler et al. [13], with permission.

diminished GH release from the pituitary and low levels of GH-dependent serum markers like IGFBP-3 or ALS. This ultimately results in a lack of somatotrophic stimulation in all peripheral target tissues. Importantly, the average lifespan of these mutants was significantly increased (fig. 1), while growth was retarded and glucose metabolism moderately impaired [13]. Together with previous longevity phenotypes, these findings suggest that longevity in mutants with impaired GH and IGF signaling may be adaptive. As suggested above, neuroendocrine plasticity allows individuals to adjust growth and body size, but also metabolism and lifespan to environmental conditions, represented in the wild by the continuous fluctuations of natural resources. Such a mechanism requires individual developmental plasticity, in particular at the level of the somatotrophic axis and the hypothalamic regulations. Previously, it had been shown in *C. elegans* and *Drosophila* that insulin-like signals in the nervous system can modulate the survival of the organism in a non-cell autonomous manner [14–19]. Conditional IGF-IR gene targeting in the mouse CNS as described above confirmed that similar mechanisms exist in higher organisms. Interestingly, other neuroendocrine hormones regulated by the pituitary-hypothalamic complex, namely the gonadotropic, thyrotropic and adrenocorticotrophic hormones, were unaffected, underscoring the specificity of this phenotype.

One of the consequences of GH deficiency in this mutant mouse was an increase in the size of subcutaneous adipose tissue (while all other tissues

were slightly diminished in size). This is most likely a direct consequence of decreased GH action on energy metabolism in adipocytes, in particular via decreased lipolytic action, leading in fine to an altered lipid profile in serum. Furthermore, GH and IGF insufficiency produced slight glucose intolerance indicative of insulin secretory defect and peripheral insulin resistance. Since these metabolic traits are normally not associated with increased lifespan, other mechanisms must be involved in the observed increase in lifespan.

Early Postnatal Diet Conditions Adult Somatotrophic Tone

Postnatal plasticity in neuroendocrine development of the somatotrophic axis can also be triggered by modifications in early diet, namely by different levels of calorie restriction (CR) during the first weeks of postnatal development. This has recently been shown in our lab by Kappeler et al. [20], who submitted newborn mice to different levels of restriction of mother's milk feeding. Underfeeding during the first 2 weeks of life significantly diminished the individual growth trajectory. More importantly, if underfeeding was limited to the milk feeding period and the same mice were fed ad libitum thereafter, they did not recover from the initial growth retardation and conserved the smaller body size for the rest of their life. The reason for limited catch-up growth, as we could show, is the particular hypothalamic-pituitary response initiated by dietary changes. Depending on mother's milk feeding in the neonates, their hypothalamus releases GHRH promoting the growth of somatotropes in the developing pituitary gland. After just 2 weeks of diminished (or increased) calorie feeding, substantial differences exist in terms of GH secretion and circulating levels of IGF. Importantly, these differences are not compensated during late development, but are progressively fixed during adult life of these animals. Kappeler et al. [20] could furthermore show that the extreme levels of somatotrophic tone in adults (groups with very high, or with very low GH) were associated with hypertension, cardiovascular risk and metabolic dysregulation. Thus, it seems that early feeding pattern change the individual growth trajectory via differential regulation of the somatotrophic axis, and that the particular individual neuroendocrine pattern can determine adult and late life pathology.

These experiments show that somatotrophic development is highly plastic and that it is possible to inhibit this hormone axis efficiently on the level of the hypothalamus using targeted mutagenesis or dietary changes [13, 20]. Subsequent reduction in IGF and GH signaling not only extends the mean lifespan, but also diminishes the interindividual variability of lifespan to a considerable degree (fig. 1). This raises the question why genetically identical wild-type mice (i.e. the control group) show a large interindividual variability in lifespan in the first place. We hypothesize that these differences in somatotrophic development occur physiologically due to environmental factors that condition the individual neuroendocrine development and trajectories. Initially

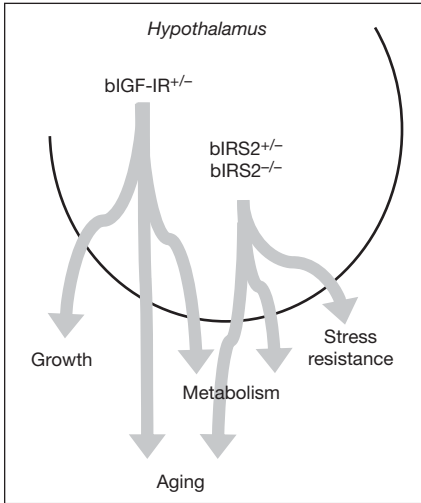


Fig. 2. Partially overlapping phenotypic consequences of brain-specific IGF-IR gene inactivation [13] and brain-specific IRS2 gene inactivation [10].

small stochastic differences are possibly amplified due to competition among siblings. Consequently, the individual rate of aging must be highly variable, too, depending on the individual's early life history. Yet, the role of insulin and IGF signaling in the brain goes beyond this regulation of the somatotrophic tone, as has been illustrated by recent work by Taguchi et al. [10] looking into the phenotypic consequences of IRS2 knockout specifically in the brain. These authors made heterozygous and homozygous brain-specific IRS2 knockout mutants and found significantly increased lifespan in both of them. The heterozygous $bIRS^{+/-}$ mutant showed in addition to that an enhanced glucose tolerance and sensitivity to insulin. Both mutants had increased adult bodyweight later in life due to an increasing amount of fat tissue. Body length was normal in the long-lived heterozygous mutant, but resistance to oxidative stress was increased under certain conditions [10]. Comparing brain-specific inactivation of IGF-IR and IRS2, it appears that both genotypes generate an overlapping, but also different hypothalamic pattern of metabolic changes, stress resistance phenotype and longevity (fig. 2). Together, these findings suggest that metabolic regulations may play a more salient role in the longevity of neuroendocrine origin.

Treatments That Extend Lifespan

Long before genes modifying aging and longevity were discovered, CR was used as an efficient means to prolong the lifespan of rodents. Maximum effects

were obtained with restriction to about 50% of ad libitum dietary intake, which is just enough to prevent malnutrition. CR strongly diminishes insulin secretion and also reduces the circulating IGF-I levels. However, the relationship between IGF and insulin signaling, CR, and aging is still not very clear. Some studies conclude that pathways responsible for CR-induced longevity are not identical to insulin and IGF pathways or only partially overlap [21], while other reports conclude that somatotrophic signals are essential for relaying the life-prolonging effects of CR in mice [22]. Whatever is more pertinent, insulin sensitivity appears to be a major factor influencing longevity in laboratory rodents. Finally, somatotrophic signaling cascades offer multiple targets for pharmacological inhibition. mTOR (mammalian target of rapamycin), a regulator of cell proliferation and cell survival, is one of them, and recently a large study administering rapamycin in adult mice demonstrated that chronic treatment with rapamycin can extend their lifespan [23]. It is possible that some of the positive effects on survival are due to decreased IGF action; however, rapamycin also affects several other pathways that may influence lifespan through alternative mechanisms. It is tempting to test this substance as a potential inhibitor of aging in humans, but rapamycin acts also as an immunosuppressor that may produce unwanted effects under long-term treatment.

Somatotropic Hormones and Human Longevity

Research into longevity using distant species showed that a conserved set of homologous genes control lifespan throughout evolution. These genes are part of the endocrine system in higher vertebrates, and an obvious question is whether they are also implicated in human longevity. While most of the available results in this area are descriptive, and some of them merely circumstantial, other findings allow cautious extrapolation to human aging. There are several reports on human growth and aging that suggest a negative correlation between mean body height and life expectancy. Such effects, however, if they exist in human populations, are currently more than compensated, e.g. by constant amelioration of hygiene, nutrition, health care and disease prevention. Under modern living conditions, average body length and life expectancy generally increase in parallel, a fact that masks any underlying reciprocal relationship. Meanwhile, several genetic studies show a correlation between human lifespan and variants of alleles that modify insulin and IGF signaling [24, 25]. One of these studies showed in a prospective cohort of aged females that certain alleles of GH, IGF-I and IRS-1 predict shorter adult body length and at the same time correlate with better survival at an advanced age [25]. In that study, GH gene variants alone explained a 2 cm lower body height and a significant increase in late life survival. Moreover, in early 2008, Suh et al. [26] reported several non-synonymous mutations that they had identified in a human population of centenarians [26]. Both mutations affected

functionally relevant domains of the IGF-IR molecule and were therefore serious candidates that could affect IGF signaling and human longevity. When the authors extended their research to a larger cohort, they found more carriers for the mutation among those with exceptional longevity, but also one carrier in the control population. This suggests that functionally relevant mutations in the IGF-IR gene may help to survive until an advanced age, but that such mutations are not sufficient to ensure longevity. These authors also found additional typical traits of IGF-IR inactivation in the group of carriers of these mutations, i.e. reduced mean body height (measured at the time they were young adults) and increased circulating IGF-I. It is of interest that in acromegalic patients persistently high circulating levels of IGF-I are a strong predictor of premature death [27], and that high circulating IGF-I in elderly people is correlated with increased mortality [28]. Historically, life expectancy of acromegalic patients suffering from pituitary gigantism (nowadays treated early to prevent pathological growth) was severely diminished compared to the normal population. Finally, another recent study found that allelic variants of FOXO3A, a transcription factor acting downstream of the GH/IGF-I pathway, are associated with increased longevity in a Japanese cohort of male centenarians [29]. These findings were confirmed and extended using a large group of centenarians from Germany. Certain polymorphisms of FOXO3A were again associated with very old age, and this association increased from nonagenarians to centenarians [30]. This was true for both sexes, and the authors were able to reproduce their results in a French cohort of centenarians, where a similar trend prevailed. Collectively, this indicates that FOXO3A is susceptible to act as a longevity gene in humans.

Taking all this evidence together, it appears that it is more important than ever to intensify the study of the role of nutrition and somatotrophic signaling in human health and longevity, and to further develop knowledge-based counseling with respect to early diet, growth regulation and human aging.

Acknowledgments

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Discussion

Dr. Stettler: For those of us who don't work with mice, can you tell us what mice die of when they get old, the same diseases as humans or different diseases?

Dr. Holzenberger: The panel of pathologies in aged mice depends on the mouse strain. We are working with F1 hybrid strains that show a large spectrum of diseases. They die of cancer, infections, cardiovascular diseases or degenerative diseases. Some mouse strains die of just a few specific diseases. So, the answer is yes, mice die of essentially the same large variety of age-related diseases as humans.

Dr. Stettler: So those are closer to humans, and if I understood correctly in your experiment where you underfed and overfed both, the overfed and the underfed had metabolic and cardiovascular risk factors. But despite those cardiovascular risk factors, the ones that were underfed did live longer, so what did they die of? Did they not die of the cardiovascular disease and metabolic disease?

Dr. Holzenberger: It is not easy to answer that question because statistics on end-of-life pathologies cannot be established routinely. We handle about 15–20 mice per group, and statistical power in terms of pathology at the time of death is low at that sample size. To perform valid statistics on pathology, we would need 100 or more individuals per group. So, concerning the glucose intolerance that these mice display, it may well be that they sense their metabolic problems and that they respond with altered feeding behavior, and thereby avoid complications. But we do not have definite proof of that.

Dr. Klisch: Every time I hear an aging talk like this, I get depressed. As the tallest man in this room and possibly the oldest man in this room, do you have any hope for me?

Dr. Holzenberger: Every time I give an aging talk I get this question, so, yes, there are exceptions to the rule.

Dr. Fasano: I guess that we are taller and bigger than our grandparents and our great-grandparents because we stimulate this growth hormone-insulin IGF axis. On one hand we grow better because we have more nutrients, but we are giving up that 20% 'buffer zone' when we push this pathway to its limits. And related to that, I have a very good friend of mine who has been devoting his science to the positive impact of limited calorie intake on longevity, and he insists that if I eat 1,500 calories or less, I will live 6 years longer. So, you put in question the findings in primates.

Dr. Holzenberger: The case of the primate is not settled, since there are contradictory results. The specific problem with aging studies in primates is that they are very long-term studies, lasting over 40–50 years. And I must confess that I am myself not on dietary restriction. Today, there is also less consensus than before on the links between dietary restriction and longevity, also because of recent work where about 40 mouse strains have been submitted to dietary restriction [1]. That work revealed that only a few of the tested strains responded positively with extended lifespan, while many others did not. Specifically, those strains that were naturally long lived did not further extend lifespan under dietary restriction, but some of the short lived did. So, somehow there must have been a selection of the mouse models that researchers used for dietary restriction experiments in the past. In other terms, not every mouse strain responds positively to dietary restriction, and we hypothesize that there are modifier genes for the dietary restriction phenotype. To my thinking, it is too early to decide on whether dietary restriction works in humans, and we still have to find out what the conditions are under which humans would respond to caloric restriction with some extra years.

Dr. Fasano: What about the human evolution?

Dr. Holzenberger: Due to the mechanisms that I described in the mouse, we observe a secular growth trend in humans, with increasing body size and body frame

over the last 200 years, in all developed countries, but we do not know what this does to life expectancy. Of course, life expectancy has increased in all developed countries, and this is due to what I showed at the very beginning with the human survival curves. We not only eat more and grow faster, but we have also much better health care, hygiene, etc., and life is generally healthier and that makes us live longer. But there may be an undercurrent in all this: although we already live longer than before, we could live even longer than that. If you think of how very old people look like, those who reach 100 years, they are rather small people, and this does not seem to be loss of body mass during aging, but a smaller body frame from the beginning. So, I think we should listen to the few studies in human populations that demonstrated that a lower body size is correlated with increased lifespan. Obviously, more systematic studies need to be performed.

Dr. Haschke: To follow up on your comment, this 'US hunger study' in healthy adults is described in one of the *Nestlé Nutrition Workshop* books. The volunteers reacted to long-term caloric restriction with depression, but all metabolic parameters – such as insulin sensitivity improved further even in healthy adults. My question to you is: are there any data on life expectancy in IGF-I-deficient populations?

Dr. Holzenberger: There are very few people with IGF deficiency, in terms of mutations?

Dr. Haschke: Yes, but in the meantime the IGF-I treatment (for children with short stature who don't react to growth hormone treatment) has become a business. My question is: are there any reliable data on morbidity and life expectancy of those cohorts? Or are there data on Pygmies?

Dr. Holzenberger: It may be difficult to conclude from data on Pygmy populations since their survival relates very much to their particular environment. But there are growth hormone-deficient humans with mutations in either the growth hormone gene or its receptor. Still, the results are contradictory. A new study on GH-deficient men and women from Ecuador reports that there is no difference in terms of lifespan. However, these people are protected from cancer and diabetes [2]. At the same time, they show a high mortality related to alcohol abuse and accidents. One may speculate that if their lifestyle would not expose them to these killers, then the protective effect of growth hormone deficiency could be more penetrant. To my thinking, it is possible that in humans some form of growth hormone deficiency may increase life expectancy, but we cannot directly transpose findings from animal studies to humans.

Dr. Lack: So, if you had a small child, would you allow your child to get growth hormone replacement, and are there any issues there? Secondly, where does exercise fit into this model, so what happens to athletes like Olympic swimmers or boxers who eat huge amounts of calories, have huge muscle mass, do they die earlier?

Dr. Holzenberger: There are few studies on that, not very systematic though, on athletes with larger body size, and they do have significantly shorter lifespan. Growth hormone abuse is a major risk factor in this context. Then, there are patients who have been long-term substituted with growth hormone, and those who displayed high GH levels under substitution had indeed an increased mortality later on. So, there are some indications but not yet sufficiently large and prospective studies, but there will certainly be more data due to the growth hormone that is sold over the internet, and the many people using it without prescription. This will create a huge amount of data in the future.

Dr. Simmer: I wanted to ask you about gender differences. I noticed on one of your slides that in the IGF mice the males lived 100 days longer, but it didn't seem to have any effect on the females.

Dr. Holzenberger: There is a lot of sex dimorphism in longevity studies studying insulin and IGF signaling, and we don't know the reasons for that yet. In the first

mutant mouse that I showed, we see significant lifespan extension in the females and only half the effect in males, and this latter effect was actually not significant. We repeated this with a slightly different mutant, which leads to just 30% inactivation of IGF receptor. Under these conditions, we see significant lifespan extension in the males and not in the females. So, somehow there seems to be a dose-response relationship that is sex dependent, suggesting that what is good for females in terms of lifespan extension is may be not good for males, and vice versa. From the last data that I showed you, the ones on early postnatal diet, it should be clear that the effect exists in males and not in females, so there is also a sex dimorphism, but for the time being we do not have sufficient data to say something about the underlying mechanisms.

Dr. Mohanty: Our observation is that postmenopausal women do much better than men, and they live longer than men. Importantly, I was involved in a small experiment in London that was about infection and nutrition in animal models. We injected the malarial parasite *Plasmodium berghei berghei* to the rats that were fed different protein levels. We found that the rats fed higher levels of protein developed jaundice, got very sick and died very early, whereas those on low levels of casein recovered from the disease, had a longer life, and were fine. So in that context, were the animals in your model more resistant to infection because of the restricted diet and that's why they lived longer, or is there some other explanation?

Dr. Holzenberger: Maybe I'll start with infectious disease, or better: the resistance to infectious disease. In these mice, if we use different stressors, and that can be paracetamol, paracetamol, diquat, or hyperoxia, we observe better survival in the mutants. So, there must be a rather broad protection from a variety of stresses, and this may be due to p66Shc signaling or FOXO signaling that in turn regulate many other downstream genes involved in stress resistance. Whether this has impact also on resistance to infectious disease, I cannot say, we would need to inoculate them with a suitable agent. This relates to the question about pathologies later in life, and although we did not obtain significant results so far, we see that specific disease categories and multimorbidity are reduced in mice with reduced IGF and growth hormone signaling. So, it could well be that the infectious diseases are less of an issue in these mutant mice than in the controls.

Dr. Harding: What would be of most interest in terms of effects in humans would be to follow up the randomized trials of growth hormone treatment of growth-restricted children. Growth-restricted children grow slowly and have low IGF-I levels, and quite a lot of them have been treated in randomized trials with growth hormone. So, they would be an interesting group to look at to answer the question.

Dr. Holzenberger: We should make a distinction between those who received growth hormone treatment because they are severely growth hormone deficient, and others who do not have such clear indication for treatment. Also, some adults with significant growth hormone deficiency should be candidates for hormone substitution. More problematic are those cases where parents come to the pediatrician and seek growth hormone treatment for children who are not even 2 standard deviations below the mean of their age group. In these cases, the indication is more facultative, and negative potential side effects in terms of possible reduction of lifespan must be taken into consideration. So, I think one should make a distinction between clearly pathological cases of growth hormone deficiency and growth retardation that need treatment, and those cases where this is more an elective treatment chosen to enhance pubertal growth. Those children are often within the normal range of body size for age, and thinking about our mouse studies, it should be taken into consideration that there may be significant effects on longevity. Within this group, I would recommend to be very prudent with growth hormone therapy. In practice, the decision will be left with the parents, who clearly need full and unbiased information about growth, hormones and effects on longevity.

Dr. Harding: My question was about the rather intriguing data you presented about both the increased and decreased feed groups having similar metabolic phenotypes but different longevity. There is evidence of a J-shaped relationships between size and many metabolic phenotypes in the epidemiological studies, and I just wondered if you or anybody else in the room knows of any evidence that there are in fact those two populations in the human studies? In other words, have we got two populations of adults with metabolic disease, only one of which is actually going to die from this?

Dr. Stettler: There is an increasing literature on healthy obese, and I think that may be what you are talking about. There is a sub-category of people who are obese but seem to be metabolically OK.

Dr. Harding: I was really referring to the people who have metabolic abnormalities, but only some of whom are actually going to get into trouble with these. If the mice studies are relevant to the human, I think it would be interesting to think further about that in terms of the epidemiology.

Dr. Shreffler: Are there inducible knockouts in genes that would be helpful for exploring these pathways in adults?

Dr. Holzenberger: We are indeed switching to inducible models, because as I showed there are developmental defects due to the lack of IGF receptors during development. For instance, in the IGF-IR knockout targeted to the forebrain neurons, if we do that in an inducible manner, we obtain the same neuroendocrine response and phenotype, proof that there is acute endocrine regulation. Therefore, it appears that there is neuroendocrine feedback that leads to increased somatotrophic tone. The interesting question then is, can we have a negative effect on life expectancy in this model when we trigger growth hormone oversecretion late in life. Then, we may also address the question whether we observe acute effect on survival or whether these are long-term effects of high growth hormone and high IGF-I.

Dr. Klish: The other side of aging research has to do with programmed cell death, apoptosis and what controls it. Do you have any knowledge within your models as to what is happening to that side of the aging process?

Dr. Holzenberger: I apologize, I have no data that could answer this question.

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Concluding Remarks

I would like to thank the audience for their participation in the discussions. We have left a lot of time for questions, and the participants from all continents were very active. The questions raised were very relevant to their own situation. That was wonderful, and I would like to thank *Jose Saavedra* for co-chairing this session, which was great fun.

Jane Harding started with the basics, as she said. She showed us which models are applicable to human research. She stated that animal models are not models for humans, but that one can learn from reactions of different species and find out whether the same can be observed in humans. There is a distinction between fetal and maternal metabolism. Fetal nutrition is directly linked to fetal growth and subsequently postnatal disease risk. Another important lesson I learned is that a change in micronutrient intake before birth has long-term consequences. Weight gain might not be the best marker, and body composition might be better. Some people in the audience actually stressed that point. The effects of different micronutrients, i.e. glycine on blood pressure, taurine on pancreatic function, and calcium (do not give too much, but be aware of effects of undersupplementation as well) also on blood pressure, are striking. The timing of nutritional changes, e.g. periconceptual undernutrition, might result in early deliveries and all kinds of metabolic changes. *Jane Harding* raised a large set of questions which will keep us occupied for a long time.

Elisabeth Novak from Canada gave a marvelous presentation. She showed us the functional role of different fatty acids in different organs. To give you some highlights: the influence of maternal fatty acid status on the fetal status, the influence of omega-3 fatty acids on appetite behavior, increased omega-6 fatty acids and decreased omega-3 fatty acids in our diets, with all the different consequences of that, lower DHA might for instance influence the neuronal migration pattern, which to me as a neonatologist is an important issue. So, thank you very much for sharing your work with us, you did a marvelous job.

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I was talking about amino acids and proteins, and I hope I showed you how important early nutrition is on later outcome in premature infants. In premature infants, nutrition can sometimes have direct and sometimes indirect effects on later outcome.

Karen Simmer showed us the knowns and unknowns of human milk banking. Indeed, 20 years ago it was very popular all over the world. In my own experience (setting up a milk bank in the Netherlands), I noticed that many people were raised with donor milk, especially during the last part of World War II. The milk banks were completely shut down during the HIV epidemics, and now they reappear. I congratulate you for the professionalism with which you described the setting up of a milk bank together with the guidelines. You have set the stage basically, and I think that many people around the world will benefit from your effort.

Finally, *Hania Szajewska* talked about systematic reviews addressing the question whether probiotics should be used, and if so, when. It was interesting to see that about 20% of the audience would advise parents to give probiotics to normal-term infants, 50% would do so in preterm infants, and again about 50% would do that in a high-risk population in their own units.

This session was a truly multinational effort to understand the effects of nutrition or supplements, such as probiotics, on later outcome. These effects might be simulated in animals, but in the end human research will help us most in determining effect size. Clearly, effects are different when the nutritional interventions are timed at different developmental stages. Interventions around the time of conception might result in other effects than similar interventions later in life.

Hans van Goudoever

I would like to thank all of the speakers for their most interesting presentations and the discussions that followed. These highlight the paradoxes that confront us with global public health nutrition issues today. Obesity is increasingly prevalent in both developed and developing nations, and yet hunger and food insufficiency remain highly prevalent across the globe in both wealthy and resource-constrained countries. Thus, hunger, malnutrition and obesity co-exist in a single population or country, and understanding the causes becomes extremely important in devising the optimal public health approach.

The 2008 FITS study provides an impressive view of the most recent food consumption data among young children in the US. Fruit and vegetable consumption is pretty clearly not meeting current recommendations. In contrast, breastfeeding duration has increased, although we are still not meeting the millennium goals. We do know from data that weren't presented yesterday that the rate of increase in obesity among 0- to 5-year-olds in the United States seems to have leveled off, which is very encouraging, and as the data

from the FITS study are further analyzed, we may begin to have some insight into the nutritional contribution to those trends.

India, like the US has impressive resources and is a highly developed nation in many ways. As *K.N. Agarwal* pointed out, India is a nation that exports food and is highly food sufficient. Yet, the prevalence of nutrient deficiencies, such as iron deficiency, is astounding. The data on iron deficiency from one of the cohorts that was presented are derived from a population of children living in the neighborhood of a major medical school and medical center. This demonstrates the public health paradox as dramatically as I have ever seen it displayed. In a high-technology environment where food is very available to many, some children continue to be subject to severe nutrient deficiencies. This reinforces that we all need to be engaged in social policy development and promote social policies that will make food available to those who are most in need of it.

Russia, another highly developed nation with advanced technology, reports a fairly small prevalence of hunger but a rising prevalence of obesity, particularly in childhood. There too, however, is an unusually high prevalence of anemia and perhaps other micronutrient deficiencies that were described by *Alexander Baturin*. This again reinforces the need to support not only appropriate social policies but effective programs that can address these nutritional issues.

One approach to addressing micronutrient deficiencies is the program designed and described by *Stanley Zlotkin* that provides 'sprinkles', a micro-encapsulated supplement that can be sprinkled on food and provides the micronutrients missing from the usual diet in a way that's easy and acceptable to families and children. Thus, this is a culturally acceptable program that is unique and has been shown to be a highly effective way of delivering micronutrients as well as nutrition education to children and families.

The last two presentations of this session addressed the issue of 'sensitivities'. *Julie Mennella* discussed how taste sensation develops over the period of infancy and childhood, and the fact that there seem to be some unique periods of time in life such as early in infancy, when there are opportunities to influence taste perception and the acceptance of specific flavors later on in childhood. The molecular biology of taste perception and the evolution of these pathways during infancy and childhood are extraordinarily interesting and important. We know that taste receptors are present not only in the mouth but in the gut as well. Understanding the physiologic effects of these receptors in the gastrointestinal tract is the subject of significant ongoing research which will enlighten our understanding of satiety and satiation. We also learned that taste perception or preference is perpetuated over time, not only by the exposure to the flavor(s) but by the context in the environment that the tastes are presented. One could conclude that having your mother threatening you with a penalty if you don't consume your vegetables may influence your taste preferences for vegetables in a different way than watch-

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ing Sesame Street, seeing Grover eating vegetables, and then being given the vegetables with your lunch and being encouraged pleasantly to eat them.

In the final presentation of the session, we heard about the very complex pathways that lead to immune tolerance and how these evolved over time to downregulate the potential of adverse immune responses to allergens in our diet or in our environment. Thus, the normal progression of immune-mediated responses to potential dietary antigens present in all foods leads to a regulatory process that controls and prevents an adverse immune response. In allergic individuals, the dose of antigen, the timing of exposure during the life cycle (prenatal, postnatal, infancy, childhood, adulthood) and the chemical structure and composition of the putative allergen help to govern whether or not the response develops into a clinical allergic response or if the immune system develops a tolerant response to these antigens so that we are not constantly reacting to various substances in the diet and the environment.

Thus, I've presented a very brief review of the presentations and discussions from this session. I have enjoyed it tremendously, and appreciate all of the work and effort that the speakers gave to their presentations. I particularly appreciated the questions and subsequent discussion that I heard from so many of you in the audience. This has been a great learning experience for me, and I think this kind of interaction between experts from all parts of the globe has so much more significance because of the diverse experiences represented here. I want to extend my thanks to all of you for your participation.

Ronald E. Kleinman

Well, we had a whole day and 6 wonderful lectures and discussions; it is now my difficult task to summarize it all.

Gideon Lack told us about the increased rates of food allergies in children, and this is apparently a worldwide phenomenon. He also told us that the time-honored principle of food allergen avoidance for the first year of life and beyond may no longer hold true. In fact, the latest statements from the UK and even from the USA appear to be now much more doubtful about recommending delayed introduction of potential allergens. On the contrary, there is emerging evidence that having early exposure to allergens might be beneficial if this occurs via the oral route as it might actually lead to tolerance. If the exposure occurs through the skin, especially when the skin is more readily available to increase permeability due to inflammation, this may actually predispose to development of allergies. Therefore, strategies to prevent sensitization might include treating eczema, eliminating the environmental allergens that we heard tend to persist for a long time, and possibly early oral exposure to the allergens. Of course, it won't be until the next 2–3 years when we hear the results of the LEAP and the EAT studies that we will know for sure whether this is really the right way to follow, but so far the evidence seems to be amounting in this direction.

Leann Birch stressed very important findings on the early development of taste in children. Infants and young children are wired to like sweet and salty foods and dislike bitter and sour ones. Such choices were desirable when scarcity of food for prolonged times was commonplace; but now that we live in an 'obesogenic' environment, they are no longer valid. *Leann Birch* showed that we may actually influence our infant's choices very early on by a number of mechanisms, for instance by repeating the exposure to foods initially rejected even for 15 or 20 times so that they become familiar with such tastes and eventually accept them. Several methods can be employed to achieve this familiarization, including presenting new foods with a positive reinforcement like attention from adults or music, etc. The most important message was that the dietary transition that occurs in the first 2 years of life is a period of a tremendous opportunity for primary prevention.

Alessio Fasano told us a number of interesting, exciting things. We learned that there are up to 27 additional genes outside of the HLA region that seem to contribute to the predisposition to celiac disease, and the time might never come when we will have the final complete genetic mapping of the predisposition to celiac disease. He then illustrated the timing of the various phases of the reaction to gluten that occurs in celiac patients. First the epithelial interaction which happens within hours, followed by the innate immunity response (both phases not genetically restricted), and then finally the adaptive response which occurs over a longer timeframe. He also commented on the importance of the timing and the amount of gluten introduction into the diet of infants predisposed to celiac disease, reminding us that it's now a given that introducing gluten during the first 3 months of life facilitates the development of celiac disease, while we still don't know exactly what would be the best time window. There are very limited data from Colorado that delaying gluten introduction past 7 months may be risky, but there seems to be now some preliminary evidence (from ongoing studies in Italy and from a multicenter study he is leading in the US) that actually gluten introduction after the age of 12 months might be beneficial. I think, however, that the most important part he focused on is the importance of microbiome. This is truly a quickly expanding field: in the past few years we have seen a number of papers [1–7] showing that the microbiome in celiac children is different from that of the normal population. This is not the result of the inflammation because this holds true even after a long time on gluten-free diet. So, there is a hint there that the interplay between the microbiome and the mucosal immunity might be exploited in order to prevent celiac disease.

Nicolas Stettler told us about the importance of diet and later development of obesity. This is of course a very hot issue in all the western countries where the obesity epidemics seems to be out of control. There was an interesting discussion on the methods of measuring body fat: BMI seems to be the most easily available, but it has its limitations; the DXA scan has even more limitations as *William Klish* also pointed out; MRI could be useful, and measuring

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the skin fold, although easily accessible and time honored, is prone to a number of errors. An important issue was commented by *Nicolas Stettler*: the association between duration of breastfeeding and later development of obesity. The preventive effect of breastfeeding, something we all gave for granted, was instead challenged by a prospective intervention study done in Belarus where actually such a correlation was not clearly found. The importance of early feeding (in some cases even feeding in the very first week of life!) on the subsequent development of obesity was well illustrated by a number of studies, opening up meaningful scenarios for a preventative intervention.

Graeme Jones brought us to Tasmania and its beautiful landscapes. His studies on a stunning 20% of Tasmanian children seem to be quite representative of the whole population; we thus learned how maternal diet during pregnancy affects the long-term bone mineral density in the offspring at the age of 8 and even at the age of 16. It is also interesting that maternal diet high in fat bore a negative and lasting effect on bone mineral density.

And finally, last but certainly not least, we were brought back to mice and dogs with *Martin Holzenberger* who told us about longevity and introduced the concept of our 'disposable soma'. Dietary restrictions in animals extend longevity, and the data were fascinating: IGF-I receptor, growth hormone and insulin appear to have a complex interplay that not only influences our metabolism and our nutrition, but also our life expectancy.

In the end, I would like to try to have a kind of 'super synthesis' of what was said so far, and especially today, so to come up with some messages which may be worth thinking about. Perhaps the most crucial message is that maternal diet is important. Far-reaching nutritional consequences of maternal diet during pregnancy may not have been so obvious before these presentations: think of what we have learned about the negative and prolonged impact of excess fat in mother's diet. The implications not only impinge on the child's future dietetic choices, but also on his/her future bone health. After birth, the preferred nutrition once again is by breastfeeding; but if this is not possible, perhaps one should give serious considerations to lower caloric feeds during the first week of life, especially in light of our 'obesogenic' environment. What mother eats continues to be important after the baby is born, if she is breastfeeding. In fact, nursing mothers should be encouraged to eat fruits and vegetables, as we have learned that this may well predispose the child toward liking such foods, something really important if one looks at the dismal data on consumption of fruits and vegetables in our young children. The infant's growth must be carefully monitored, as an excessively fast growth may imply not only higher risk of later obesity but also possible shorter lifespan. Many more important conclusions could be drawn from such splendid workshop, and surely we all enjoyed the sharp presentations and the lively discussions throughout these exciting 3 days. Much has been learned, much more needs to be unraveled. Let's all go to work for the future of our children!

Stefano Guandalini

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