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Preface

Human milk is a complex biological fluid that contains all of the essential nutrients as well as other functional components that are thought to contribute to the short- and long-term health outcomes of breast- versus formula-fed infants. The goal of this workshop was to review the current evidence for the composition of human milk and its effects on the developing infant, to identify existing knowledge gaps, and to suggest future opportunities for research in human milk and lactation.

The first session set the stage with speakers providing a historical perspective of the place of breastfeeding in medicine, a biological perspective for the role of breastfeeding in infant health and an overview of the physiological basis and mechanics of breastfeeding. It is a goal of many organizations, governments, and health professionals that babies should be breastfed for at least the first year of life and exclusively for the first 6 months. Thus, breastfeeding may be regarded as major global public health intervention. In general, public health interventions should be rooted in sound scientific evidence. Unfortunately, in the past some of the key scientific pillars that have supported this important field have been based on flawed science and mistaken biological thinking. It was one goal of this session to identify such flaws in order to help pave the way to a new evidence-based “breastfeeding medicine” for the future. A key premise of this session was that a firm scientific evidence base in this rapidly developing field would have practical implications for the care of breastfed babies which in turn would be in the interests of population health. Breastfeeding itself is a mechanical process; therefore, the success of breastfeeding as a public health intervention depends on successful suckling, and for those that assist professionally with breastfeeding management, best practices are underpinned by a scientific understanding of the mechanics of suckling. However, despite the intensity and complexity of a new wave of current research into the mechanics of suckling, this work did not displace the long-standing best practices in breastfeeding management, derived from historical studies. Session 1 as a whole emphasized the
importance of getting the science of breastfeeding right, the need for relevant health professionals to understand this science and the great potential that breastfeeding has, as a branch of medical practice, for influencing short- and long-term population health outcomes.

Next session has presented an update on our current understanding of the composition of human milk components and their potential physiological benefits. In the past decade, our understanding of human milk composition has rapidly advanced through the application of sophisticated, high-throughput analytical tools. Infant nutrient requirements are largely based on nutrient intakes of breastfed infants, which are generally assumed to be adequate. Information on nutrient concentrations in human milk and how they may be affected by various factors, such as maternal stores and diet, ethnicity, and length of lactation, is therefore imperative. This is particularly important for micronutrients as they have been difficult to analyze, and micronutrient deficiencies may have short- and long-term physiological implications for infant growth and development. Recent studies performed at multiple geographical locations and with adequate sampling methodology and analytical methods provide essential information for understanding requirements and establishing better recommendations. The composition of human milk proteins, fatty acids, oligosaccharides, and fat- and water-soluble vitamins was presented, along with emerging evidence on human milk microRNAs and exosomes, which may constitute biological messengers affecting infant development. Improvements in dairy technology have enabled the isolation of bioactive proteins from bovine milk for supplementation to infant formula. Findings of a randomized clinical trial with a milk fat globule membrane fraction demonstrate beneficial effects on multiple outcomes ranging from immune and cognitive development to microbiome modulation.

Session 3 extended upon Session 2 by focusing on the clinical aspects of human milk on infant health outcomes, including growth and metabolic outcomes and cognitive, immune, and microbiome development. Overall, breastfeeding "programs" a healthier growth trajectory and reduces the risk of overweight and obesity. However, the effects of human milk are specific to the infant’s physiological state (term vs. preterm very-low birthweight) and environment (developing vs. developed country). In preterm infants, the effect of mother’s own milk (MOM) on reducing diseases associated with prematurity is affected by the dose of MOM and the timing of the exposure, suggesting disease-specific mechanisms that are impacted by MOM. Human milk contains factors that influence the development of the infant microbiota, including human milk oligosaccharides. The microbiota, in turn, shapes immune development. Components in human milk may also directly influence gut mucosal immunity and promote
immune tolerance. Recent evidence has underscored the interrelationship between the gut, microbiome, and the brain (microbiome-gut-brain axis) in cognitive development, and human milk components are key players in all aspects of this relationship. The application of noninvasive imaging techniques is providing new insight into the effect of early-life nutrition on brain structural and functional development, with links to learning and memory.

The final session reviewed the current state of human milk research, which is challenged by the complexity of lactation even with respect to defining the simple composition of milk. In addition, the full implications of emerging data on lactation as a remarkable biological process and the diversity of functions of human milk to the protection, development, and education of the infant has not been integrated into our current views about breastfeeding and lactation, particularly in the medical community. Future research is needed to build a more complete and predictive understanding of milk’s diversity, which will be accelerated by recruiting diverse scientific fields and disciplines complete with their tools, perspectives, and insights into biological structures and functions. Biological research has been revolutionized by the science of genomics and its associated global platforms of proteomics and metabolomics. It is impossible to think about complexity of human milk and lactation without evolution and anthropological aspects. The session presented a remarkably insightful anthropologic perspective to lactation in its broadest sense from molecular mechanisms to infant behavior. Additionally, lactation within the context of comparative biology was discussed. This area of research has been a powerful engine for scientific discovery by providing scientists with the tools of biology itself to understand the basic mechanisms by which living organisms are structured and function.

This workshop provided a venue in which to consider human milk and lactation from multiple perspectives, highlighting what we know, what we do not know and promising avenues for future research. It is clear that human milk is more than the sum of its isolated chemical components. Due to the importance of breastfeeding to child health, the application of state-of-the-art analytical approaches to interrogate the complex structure of human milk and its effects on the recipient infant should be a high priority in pediatric research.

Sharon M. Donovan
J. Bruce German
Bo Lönnerdal
Alan Lucas
Foreword

Human milk presents the optimal nutrition for infants and is key to sustaining health and building the foundation for growth and cognitive development. The World Health Organization (WHO) recommends that infants should be exclusively breastfed for the first 6 months of life and subsequently receive suitable complementary foods while breastfeeding continues up to 24 months of age or beyond.

The global initiative to encourage breastfeeding for all infants worldwide represents one of the most significant public health interventions. It is therefore critical that any guidance to support breastfeeding is evidence-based.

Rapidly advancing technology has allowed us a closer look at the different components of human milk and shed light on their biological effects on growth, metabolism, cognition, and immunity. This new knowledge is constantly enriching our picture of how human milk sets the foundation for health in later life. Yet, researchers face many challenges in their quest to unravel its complexities. An understanding of human milk is inextricably linked to an understanding of the biology of the growing infant. Any clinical study that aims to elucidate the effects of a specific element in human milk must overcome the double hurdle of design and outcome: how can we test such a complex substance or extract a meaningful endpoint from the intricacies of infant development? Success relies in part on obtaining a cohesive body of in vitro, in vivo, and clinical data.

The 90th Nestlé Nutrition Institute Workshop brought together the world’s experts on human milk, chaired by Prof. Sharon M. Donovan (Professor and Melissa M. Noel Endowed Chair in Nutrition and Health, Department of Food Science and Human Nutrition, Carl R. Woese Institute for Genomic Biology, University of Illinois), Prof. J. Bruce German (Director, Foods for Health Institute, University of California, Davis), Prof. Bo Lönnerdal (Distinguished Professor Emeritus, Department of Nutrition and Internal Medicine, University of California, Davis), and Prof. Alan Lucas (MRC Clinical Research Professor and Head of the Childhood Nutrition Centre, Institute of Child Health, University
College London). The four sessions in the workshop touched on the full spectrum of our knowledge of human milk, from the history and mechanics of breastfeeding, its physiological effects, to the new surprises revealed by metabolomics and comparative biology.

Although it is well accepted that the early years of a child’s life are critical for growth and development, we have little mechanistic understanding of how the infant diet shapes short-term and long-term health. One of the key learnings in this workshop is that human milk is not only a source of essential nutrients, but also contains a variety of bioactive substances. These include essential microbes, long-chain fatty acids, complex oligosaccharides, nucleotides, and bioactive signaling proteins and hormones.

We are only just beginning to glimpse at how these components protect against infections, regulate infant development, and modulate long-term outcomes. A deeper understanding of the function of human milk will also help to enhance outcomes in vulnerable populations, including premature infants, those with low birthweight, and infants with special dietary needs.

We would like to thank the four Chairpersons Sharon M. Donovan, J. Bruce German, Bo Lönnerdal, and Alan Lucas for putting the scientific program together.

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Abstract
The global drive to promote breastfeeding targeted at all 134 million infants born/year on the planet is one of the most pervasive public health interventions. It is, therefore, critical that the breastfeeding field is rooted in sound evidence. Three important scientific pillars of breastfeeding have been: (1) that human milk (HM) is the product of 200 million years of mammalian evolution; (2) that HM composition should be seen as the gold standard for infant nutritional requirements; and (3) that HM has numerous clinical benefits for the infant. I shall look carefully at these areas to help pave the way to a more solid basis for modern breastfeeding medicine. Firstly, I shall look at evolutionary theory for human breastfeeding and consider in general terms the implications for optimal nutritional care of breastfed infants. Secondly, I shall show how HM composition has been incorrectly translated into dietary intake in a large body of past flawed work that resulted in misleading data. Implementing such data as a model for infant formula appears to have increased the risk of obesity and cardiovascular disease (CVD) in formula-fed infants. Finally, most studies that examine the benefits of HM are observational and potentially confounded. So, this body of data needs to be backed by experimental evidence. Here, I shall use preterm infants as a model, since numerous RCTs and physiological studies over 40 years have compared exclusive HM feeding versus cow’s milk exposure. Unexpectedly diverse immediate beneficial effects span the field of neonatology, and long-term programmed effects have been shown for cognition, brain structure, risk factors for CVD, structural development of the heart and lungs, bone health, and atopy. These data add much weight to the evidence, obtained in full-term infants using observational study de-
Introduction

Given 7 billion people on the planet and 134 million births/year, the recommendation that all babies should be breastfed constitutes a colossal public health intervention. All public health interventions should be rooted in sound scientific evidence and I shall consider some modern advances in the science and understanding of this important field.

I shall focus on 3 important pillars in breastfeeding medicine: (1) that human milk (HM) is the product of 200 million years of mammalian evolution; (2) that HM composition is the gold standard for infant nutritional requirements; and (3) that HM has numerous clinical benefits for the infant.

Finally, I shall emphasize the great importance of breastfeeding as an evidence-based clinical and public health intervention.

Breastfeeding and Mammalian Evolution

With 200 million years of mammalian evolution, breast milk has evolved major diversity – for instance a 2% concentration of fat in mare’s milk contrasts with over 40% fat in the milk of the harp seal, where the offspring must survive extreme cold. Nevertheless, the application of evolutionary biology to human breastfeeding requires some special considerations with potential implications for practice [1].

Until relatively recently, humans lived in hunter-gatherer societies, but, in a short period, as intelligent primates, humans changed their environment dramatically, whereas our genes are still ancient. The consequent mismatch between our genes and environment is known as “evolutionary discordance.” As Cordain et al. [2] noted for adult humans, the principal phenotypic manifestation of evolutionary discordance is disease. Thus, it is proposed that the high incidence of obesity and cardiovascular disease (CVD) in modern humans is due to the mismatch between genetic adaptation and our modern diet – an example of evolutionary discordance.

The question of relevance here is whether human breastfed infants are affected by evolutionary discordance and how this should be managed to complement the considerable value of breastfeeding identified later in this article. Thus,
modern mothers eat less green leafy plants than our ancestors and presumably have less vitamin K in their breast milk [3]. This may explain the past occurrence of late vitamin K deficiency bleeding in modern breastfed infants – a condition that had a high incidence of intracranial bleeding. Thus, all babies now receive prophylactic vitamin K after birth. A further example is that a consequence of recent migration of human populations into less light-exposed areas of the globe is increased propensity to vitamin D deficiency, which may require vitamin D prophylaxis. An intriguing hypothesis to explain the occurrence of early iron deficiency anemia comes from the observation that piglets put in a concrete pen develop iron deficiency since pig’s milk is relatively low in iron and a concrete pen prevents iron intake from soil [4]. Hallberg [5] speculated that early human infants might have eaten soil to supplement the iron received from human breast milk, but with environmental change and modern public health, modern infants no longer consume iron from soil.

One consequence of the major recent change in the diet of humans is that the n-6/n-3 fatty acid ratio in the diet of hunters-gatherers is believed to be around 1:1 whereas with a modern Western diet this ratio is around 15:1, reflecting a relatively low n-3 fatty acid status in modern mothers [6]. The impact of supplementing the diet of a lactating mother with n-3 fatty acids is not established but does at least raise the hypothesis for future testing that nutritional status of the offspring might be further optimized by dietary care of breastfeeding mothers.

In summary, current evidence (see later) shows that breastfeeding is superior to its substitutes on numerous health grounds. Nevertheless, given the evolutionary aspects considered, it is in the interests of population health to identify areas in which nutritional care of breastfeeding mothers or their babies could further improve outcome – a principle already in practice in relation to the use of prophylactic vitamin K and vitamin D in infancy.

**Breast Milk Composition as the Gold Standard for Infant Nutritional Needs**

HM composition has generally been regarded as a gold standard for deriving infant nutritional requirements – for instance in situations where artificial feeding is required. This has certainly been a most helpful concept.

However, for breast milk to be a valid gold standard, it is critical that accurate data are obtained using appropriate methodology. This latter aspect is the one that is discussed in this section since it will be argued that despite intensive work on the composition of breast milk, misleading data have been derived in the past that have misdirected nutrition practice in ways that have had adverse impact on babies and their long-term health.
In 1953, Hoobler et al. [7] were able to summarize no less than 1,500 scientific publications on the composition of HM. In 1977, the UK Department of Health added further to this list: an official publication on the nutrient content of breast milk obtained by complete expression of one breast in mothers from 4 UK cities [8]. These data were proposed to provide a basis for infant nutritional needs and a model for the design of infant formulas. It was at this stage that this and past studies on breast milk composition were challenged as methodologically flawed [9].

One major difficulty in the study of breast milk content is obtaining representative samples of breast milk for analysis. Breast milk fat, and hence energy content, varies greatly during a feed, between breasts, and throughout lactation. Our own data show that during the course of a breastfeed, breast milk fat content doubles, and milk flow, statistically at least, decreases in a curvilinear manner [10]. We suggested that obtaining representative values for milk fat might be best derived by studying milk composition and milk flow during the feeding process, yet this had never been done. In the absence of such knowledge, we hypothesized that milk obtained for analysis in the traditional manner by unphysiological manual or mechanical expression of the breast – so-called expressed breast milk (EBM) – might differ greatly in its fat and energy content compared to the milk obtained by the baby during physiological breastfeeding – termed by Lucas “suckled breast milk” (SBM) [11]. This difference could occur, for instance, if expression of the breast removed more high-fat hind milk than would be obtained by the baby if the breast was not fully emptied during feeding.

In order to study SBM, a milk sampling system was devised by modifying a clinical nipple shield worn on the breast during breastfeeding. The modified nipple shield contained a milk sampling line so that milk could be sampled continuously during a breastfeed, and it also contained a flowmeter in the tip. Initial research using the nipple shield sampling system showed that SBM fat content was around 2.5 g/100 mL versus a figure of around 4.0 g/100 mL obtained in a vast number of prior studies on EBM composition [8, 11]. Thus, if valid, our data suggested that using EBM, it was possible to overestimate milk fat content by 60% compared to SBM obtained during normal feeding. We estimated the energy content of SBM to be 58 kcal/100 mL compared to around 71 kcal/100 mL based on over 1,500 prior publications. This would equate to a methodological error in measuring milk energy content of over 20% when studying EBM versus SBM.

When these data on SBM were published, they were too radically different to those published previously using EBM to be widely accepted. So, to confirm our findings on the energy content of breast milk, we used the doubly labeled water method in a novel way. In this method, 2 naturally occurring stable isotopes:
deuterium (heavy hydrogen, $^2\text{H}$) and heavy oxygen ($^{18}\text{O}$) are given orally producing enrichment of these isotopes in body fluids. Decline in these isotope enrichments back towards baseline is measured in urine or saliva over several days. The slope of the decline in $^2\text{H}$ can be used to measure water output (since hydrogen is lost as water), which in steady state reflects water intake, and, from this, milk volume intake can be derived. The decline in $^{18}\text{O}$ is faster since oxygen can be lost in both water and carbon dioxide. Hence, the difference in the decline in $^2\text{H}$ and $^{18}\text{O}$ is the CO$_2$ production rate, from which energy expenditure can be derived – and hence metabolizable energy intake. Thus, over several representative days, milk volume intake and energy intake can be estimated, and, by dividing the latter by the former, the energy content of breast milk is derived without any recourse to breast milk sampling. This approach produced values for energy content of breast milk according to postnatal age of 57–61 kcal/100 mL, thus confirming our previous values using the nipple shield system [12]. Later work has confirmed our finding that breast milk energy content had been greatly overestimated in past EBM studies.

One importance of these findings is that formula manufacturers based their products, and still do, on the composition of EBM, which emerges as the wrong model.

In addition to errors in prior estimation of breast milk fat and energy, breast milk protein content was also overestimated by using analytic methodology developed by the dairy industry. In cow’s milk (CM), there is little nitrogen that is not part of protein so that it is possible to estimate protein content by multiplying nitrogen content by a constant (6.38) [13]. This was used inappropriately for human breast milk in which there is a high content of nonprotein nitrogen (e.g., urea), which should not be counted as protein. Thus, more recent work shows that the true protein content of breast milk is significantly lower than previously thought [14], and this is one reason why infant formulas have substantially higher protein content than breast milk.

Thus, as a result of flawed methodology for breast milk compositional analysis, a generation of babies were fed on formulas modeled on EBM composition unphysiologically high in fat, energy, and protein. This constituted an inadvertent experiment in early overfeeding, and animal studies since the 1960s show early overfeeding increases later cardiovascular risk factors [15].

The higher nutrient intake of the formula-fed infants is believed to be a major factor in the faster early growth of formula- rather than breastfed infants. So, does it matter that formula-fed babies grow faster? In 2004, based on our nutritional intervention trials and animal evidence, we published our postnatal growth acceleration hypothesis, which proposes that faster early growth increases the risk for later obesity and CVD [15]. In that publication, the known in-
creased risk of obesity and cardiovascular risk markers with formula feeding was proposed to relate to the faster growth rate. Since then, over 60 studies, including randomized trials, have supported the postnatal growth acceleration hypothesis.

Thus, flaws in research on breast milk composition were indirectly partly responsible for the major modern epidemic of CVD and obesity – a salutary example of the importance of methodology in science. The field has now become a priority for research on both breastfeeding and formula feeding.

**The Benefits of Breastfeeding Revisited**

Arguably, the main platform for the global promotion of breastfeeding is the scientific evidence for its clinical benefits. However, with few exceptions, the comparison of breast- and formula-fed babies has not been based on randomized trials that would prove causation, but rather on observational associations.

Initially, the main outcomes of interest were infection and cognition, but these outcomes are potentially highly confounded by the differences in the populations (statistically) that choose to breastfeed or formula feed. As an example, cognitive benefits in breastfed babies have been described in a number of studies since 1929, but in 2006, Der et al. [16] concluded from a meta-analysis and study of sibling pairs that there was no cognitive benefit due to breastfeeding, and the previous positive findings were explained by the higher maternal IQ in those who chose to breastfeed. This study emphasizes the ever-present potential for confounding in epidemiological studies where there are major demographic differences between the groups compared, though the study by Der et al. [16] was also nonrandomized.

Today, a wide variety of beneficial outcomes has been linked beneficially to breastfeeding [17], including CVD and obesity risk, atopic disease, IQ, brain size, infection, cancer, sudden infant death, celiac disease, and type I and II diabetes – but again these beneficial outcomes have only been epidemiologically associated with breastfeeding and not determined experimentally, leaving uncertainty over causation.

The challenge then is how better-quality evidence can be obtained, given the constraint that randomized trials, for instance comparing the outcome of breastfeeding versus formula feeding, are generally precluded on ethical grounds.

**The Preterm Infant as a Model**

The area I shall focus on here is the use of the preterm infant as a model. Whilst accepting that the spectrum of diseases and the sensitivity to early nutrition is somewhat different in preterm and term infants, neonatal care is an
area where it has been ethically possible to conduct numerous strictly randomized trials of EHM feeding versus exposure to CM. My argument is that if a wide range of important outcomes in preterm human infants are favorably impacted by HM feeding, this would indicate that the weaker observational data on the benefits of breastfeeding in full-term infants are more likely to be causal – especially when the same outcomes (e.g., infection, allergy, cardiovascular risk, or cognitive development) can be studied in both the preterm and term populations.

Preterm Trials Comparing Exclusive Human Milk Feeding versus Exposure to Cow’s Milk

There are 3 categories of randomized controlled trials (RCTs) that provide evidence on the benefits of HM or adverse impact of CM.

1. Historical trials [18] comparing EHM feeding versus CM-based products (used either alone or in combination with HM). In these trials, the HM arm received no CM since this was the era before the development (in the later 1980s) of CM-based breast milk fortifiers. The largest of such trials was by Lucas et al. on over 500 infants but at least 5 other smaller RCTs of this nature were done by other investigators.

2. The historic fortifier trial of Lucas et al. [19] tested the clinical impact of adding CM-based fortifier to breast milk versus no fortification (which was ethical at this time when fortifiers were just being introduced into practice).

3. A third RCT category has a long history, as explained here. In the late 1970s, the first evidence began to emerge that HM protected against necrotizing enterocolitis (NEC) and sepsis. However, extensive research showed that HM alone did not meet the needs of preterm infants for protein and other nutrients needed to fuel the rapid growth of the preterm infant, notably the growth and development of the brain. In response to this, CM-based special preterm infant formulas were devised in the 1970s (CM-based HM fortifiers came later in the 1980s) – but by the late 1970s evidence began to accumulate that CM products had adverse effects. In response to this, Lucas et al. [20] developed the concept of lacto-engineering whereby donor breast milk (DBM) was separated and reconstituted to produce HM-based formulations enriched in HM protein and fat that met nutritional needs of preterm infants and allowed total exclusion of CM. The HIV epidemic in the 1980s closed down HM banks, but with the more recent re-emergence of milk banking the opportunity arose for commercial production of HM-based fortifiers and preterm formulas allowing preterm infants to receive an exclusive HM (EHM)-based diet. In the USA, many level 3 or 4 neonatal intensive care units have used these HM-based products providing a new opportunity to do RCTs
and quasi experimental studies comparing current practice using diets containing CM versus feeding an EHM diet with these modern lacto-engineered products.

Necrotizing Enterocolitis and Systemic Sepsis

In term infants, breastfeeding is associated epidemiologically with significant reduction in infection. In preterm infants, more serious infective/inflammatory conditions – notably NEC and proven systemic sepsis – are common, and RCTs and related studies can be used to test the impact of HM versus CM as a model. At least 7 RCTs including trials from the prefortifier era [18] and 2 trials of lacto-engineered products examined the impact on NEC [21, 22]. In all, 6 trials of HM/CM exposure were included in a Cochrane meta-analysis [18]. Collectively, the RCTs show around a 3-fold increased risk of NEC with CM exposure. Further to this, at least 8 quasi-experimental studies have been done on around 4,000 (published in full [23] or in abstract form) that examine the impact of introducing an EHM diet with lacto-engineered products; the incidence of NEC was on average 3 times higher when infants were exposed to CM compared to those fed the EHM diet. With regard to sepsis, 3 historic RCTs from India showed exposure to CM increased the risk of major infection [24]; and the quasi-experimental study on 1,600 babies before and after introducing an EHM diet using lacto-engineered products showed a major fall in the incidence of late-onset sepsis from 30 to 19% [23]. We conducted 2 further RCTs with NEC or sepsis as a combined outcome – our fortifier trial [19] and an unpublished analysis of our historic trial comparing preterm formula with DBM; in both cases, NEC or sepsis was doubled in the CM limb. The US trials on lacto-engineered products showed NEC, NEC surgery, and sepsis were dose related to the amount of CM in the neonatal diet [25], in accord with our own data from the UK [26].

Thus, at least 12 RCTs and 8 quasi-experimental studies show that HM has a major protective effect against infective/inflammatory conditions that provide support for a causal role of breastfeeding in protecting against infection in term infants.

The clinical importance of NEC and sepsis is emphasized by the evidence that these are accompanied by an increased risk of cerebral palsy and lower cognitive performance [27].

Mortality

The US trials (combined) of lacto-engineered products show that death rate was 4 times higher in those exposed to CM versus an EHM diet comprising modern lacto-engineered products [25].
Retinopathy of Prematurity

A recent RCT in Canada has been presented in abstract form based on infants with a 100% base diet of HM but randomized to a standard CM-based HM fortifier or a HM-based HM fortifier. The group exposed to the CM fortifier had a significant 6-fold increase in potentially blinding retinopathy of prematurity. In all, at least 7 further studies (5 of them included in a systematic review) showed collectively in around 4,000 subjects a major increase in retinopathy of prematurity with CM exposure compared to EHM [23, 28].

Cardiorespiratory Impact

In a quasi-experimental 4-center study by Hair et al. [23], comparing CM exposure with an EHM diet, the EHM group had significant reductions in need for ventilation, bronchopulmonary dysplasia, and patent ductus arteriosus. Assad et al. [29] found a 73% increase in bronchopulmonary dysplasia in those exposed to CM rather than an EHM diet.

Cognitive Development

In preterm infants, numerous observational studies have shown that use of HM in neonatal care is associated with higher IQ or DQ but, like the studies in full-term infants, such data do not prove causation. However, the opportunity to study this using an experimental design arose with our own RCTs in neonates whose mothers had elected not to provide their own breast milk (thus eliminating the potential confounding relating to mother’s choice to provide breast milk). These two trials compared as sole diets: (i) DBM versus preterm formula (PTF), and (ii) term formula (TF) versus PTF. The first of these trials, DBM versus PTF, compared HM with CM, but the CM arm (PTF) provided much higher protein and energy intakes. Nevertheless, the HM (DBM) group was not disadvantaged in later cognitive scores, suggesting that breast milk had factors that ameliorated the poor nutrient intake. In order to remove the major nutritional difference between these groups, we elected to compare DBM from trial (i) with TF from trial (ii) since these were diets both suitable for term infants. This cross comparison of RCTs was justified since both trials used the same PTF, thus constituting an “internal standard.” The HM (DBM) group had a significant 7-point advantage in the Bayley psychomotor index compared to the TF, providing compelling experimental evidence that HM promoted better cognitive development than seen in the CM (TF) group.

This finding is consistent with a rare RCT done in term infants – the Belarus trial – a cluster RCT done on over 17,000 mother-infant pairs. The intervention in breastfed infants was active breastfeeding promotion compared with no active promotion in the breastfed control group. A significantly longer duration of
exclusive breastfeeding was achieved in the intervention group, which showed a 7.5-point advantage in verbal IQ at 6.5 years [30].

These two pieces of experimental evidence give weight to the view that studies that show an association between breastfeeding and superior cognitive outcome are causal.

**Cardiovascular Risk Factors**

Many epidemiological studies link breastfeeding to CVD risk factors. In our large historic RCT of EHM versus CM exposure in preterm neonates, we found at the 16-year follow-up that the EHM group had favorably reduced the LDL:HDL cholesterol ratio, diastolic blood pressure, insulin resistance, and metabolic tendency to fatness (leptin resistance) [31–33]. Thus, early HM feeding in a strictly randomized trial reduced 4 key risk factors for CVD. The effect size was large; for instance, the impact of early HM feeding on later cholesterol alone would be expected in adults to reduce CVD by 25% and death by 13–14%. These data add weight to the causal nature of a protective role of breastfeeding for future obesity and CVD.

**Atopic Disease**

The relationship between breastfeeding and later atopy has been observational and uncertain. In our historic RCT comparing EHM with CM exposure, those with a family history of atopy fed an EHM diet had a major reduction in eczema, food and drug reactions, and wheezing at the 18-month follow-up [34]. Thus, strict experimental evidence confirms that HM, at least in those with a family history of atopy, is protective against future development of atopic phenomena.

**Conclusion**

Clearly, the HM-fed preterm infant is not a perfect model for the breastfed term infant, and some outcomes considered above would not occur in term infants. Nevertheless, it is a very useful model and conceptually, experimental studies in preterm infants add much weight to the view that breast milk is likely to have broad and important causal effects on short- and long-term outcomes in healthy full-term infants.

**Further Models**

Given the difficulty in providing an evidence-based underpinning for the impact of breastfeeding on clinical outcome, it is important to explore creatively further opportunities for experimental studies. In this paper, I have considered the value of RCTs in preterm infants and noted the inventive Belarus study on breast
milk and cognitive development in term infants. One potentially promising area is the use of RCTs to study the impact on outcome of individual components of breast milk, for instance, HM oligosaccharides.

**Overview**

It has been an objective in this paper to examine some of the general principles that underlie the science of breastfeeding medicine in order to help strengthen this important field, in the interests of improving infant, child, and population health. The critique in the 3 sections of this paper – on human evolution, HM as a gold standard, and the proposed benefits of breastfeeding has significant, practical clinical and public health implications. Breastfeeding and indeed the use of HM in neonatal intensive care is entering a new era of quite unexpected importance for human biology and health.

**Disclosure Statement**

Alan Lucas has taken part in educational events organized by infant food, breast milk and feeding device companies for which he has received an honorarium and expenses. He has provided medical scientific advice to breast milk companies for which he has received consultancy payment.

**References**

The Biomechanics of Breastfeeding: Bridging the Gap between Engineering-Based Studies and Clinical Practice

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Abstract
Currently accepted “best practice” for managing breastfeeding effectively (WHO/UNICEF) is largely based on a historical view of how babies remove milk from the breast, which had persisted for several centuries. The collective wisdom was verified by imaging studies in the 1950s and 1980s, to reach a consensus view – clinical management principles, based on such research, have proved highly effective. Over the past decade, the mechanics of suckling, and how the baby removes milk from the breast, have been revisited, using modern imaging technology and by the application of engineering-based techniques, which seek to develop explanatory models of how suckling works. While the imaging studies have caused us to expand our view of the process, the engineering-based models have proved somewhat contradictory, tending to undermine the new consensus. Such models are complex, mathematically difficult to evaluate, and without simple lessons by which clinicians/practitioners can update their practice. This presentation will seek to demonstrate the current agreement between imaging studies, and elucidate recent engineering-based models of milk extraction, to achieve a fresh consensus – a “revised suckling physiology.” Certain limitations of the engineering-based models will be addressed, showing why they do not yet provide a definitive explanation of how babies remove milk from the breast. The encouraging news, however, is that current “best practice” for breastfeeding does not need to be updated; in fact, a new conclusion indicates that the guiding principles are even more relevant than before.

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Introduction

For the best part of four centuries, the medical world was secure in its view of how babies fed and removed milk from the breast – the terms “sucking” and “suckling” became mutually replaceable, even though they describe separate processes. Two commonly accepted facts remain today: first, the baby generates high levels of suction pressure in the oral cavity, so that any object placed in a baby’s mouth (bottle-teat, finger, pacifier) cannot easily be removed. Second, the baby’s tongue moves in a wave-like manner, with positive pressure being exerted rhythmically by the dorsum of the tongue surface to the underside of the nipple/breast complex held in the baby’s mouth; this is regarded as a type feature of the baby, taken to indicate its neurodevelopmental maturity.

The latter tongue movements, originally identified by practical observation [1–3], were later visualized using various techniques including cineradiography [4], 2D ultrasound [5, 6], direct filming [7] and, most recently, 3D ultrasound [8]. All such methods were unambiguous in observing peristaltic tongue movements (PTMs), on the basis of which it was assumed that they played a role in expressing milk from the breast; we have since confirmed that this collective view is essentially correct [9, 10].

Two studies, principally Eishima [7] and Geddes et al. [11], identified a novel feature of infant feeding, involving a localized drawing down of the central region of the tongue, adjacent to the nipple tip. To this movement was imputed the ability to generate increased \((\text{added})\) suction at the nipple surface, claimed to play a predominant role in milk extraction from the breast; subsequent studies have extended and elaborated on these claims [12–14]. We can similarly confirm that these authors are correct in their observations, and in their proposition that the action aids milk extraction; nonetheless, some vital caveats need to be considered when evaluating the full validity of their claims.

What We Already Know

Seven principal \(\text{forces}\) are present and active during breastfeeding, the first three affect the pressure of milk within the breast; all but one is active in milk transfer, while one plays a key role in retaining the breast within the baby’s mouth.

(1) \(\text{Atmospheric pressure}\) is an important force in the process, although it is eclipsed by the positive pressure created by (2) the mother’s “let-down” or milk ejection reflex (MER). The MER creates phasic (intermittent) increases in positive pressure in the milk held within the breast, while also causing the milk ducts to dilate, so providing less resistance to the flow of milk to the nipple surface.
Because breastfeeding is such a highly dynamic form of milk extraction, atmospheric pressure is more likely to play a role in milk extraction by breast pump (being less dynamic). These two forces constitute one side of an active pressure gradient.

A less obvious process creating positive pressure in the breast is (3) the compressive pressure of the baby’s lips against the breast, pressing cyclically on the breast surface surrounding the ducts during feeding. Its role in milk extraction is not dissimilar to that of the flanged cone of a breast pump, pressing against the breast; an awareness of this subsidiary process has largely arisen from studies of breast pumping. A well-attached baby, making a wide flange at the breast, will capitalize on this, while also taking a large mouthful of breast tissue.

Within the baby’s mouth, creating the other side of the active pressure gradient, is (4) intense negative suction pressure created by the baby cyclically lowering the rear surface of its tongue. This is responsible for generating baseline suction pressure which both draws the breast into the baby’s mouth and retains it throughout the feed. This force is unstable, however, as any milk issuing from the breast into the oral cavity negates it, making it necessary for it to be reapplied in a cyclical manner throughout feeding.

These four principal forces are necessary and sufficient for a breast pump (electric or manual) to create adequate milk removal from the breast. The cyclical application of negative suction pressure at the nipple surface (aided by the three other factors) is adequate for sustaining milk collection from the breast.

The two unique features which the baby brings to the process are: (5) the compressive action of the baby’s jaws (and gums), and (6) PTMs applying retrograde waves of positive pressure to the underside of nipple surface. The peristaltic action of the baby’s tongue is obligate, playing the primary role in both milk transfer and expelling the milk bolus into the oro-pharynx for swallowing. The action of these two forces alone is not dissimilar to hand expression of the breast, which requires no negative suction pressure to remove milk. The fingers press into the breast at the base of the ducts, in a similar action to the baby’s jaws, and the opposed fingers are drawn towards the nipple end to express milk; this emulates the peristaltic action of the baby’s tongue. The role of the baby’s jaws should never be overlooked, as they effectively “gate” the release of milk, letting it enter the milk ducts, lying within the nipple/breast teat complex, in packaged bundles, rather than as a continuous outflow of milk from the breast.

Accordingly, there are two sets of forces – the first four alone are capable of milk extraction, and are employed specifically when a breast pump is used to extract milk. The next two forces alone are capable of milk expression from the breast, in the absence of the other forces. They are akin to “pump extraction” and “hand expression” and use entirely different modes of action, yet both are
effective for expressing/extracting milk from the breast (just as “hand milking” and “machine milking” are equally effective with dairy animals). The essential beauty of mammalian suckling is that these separate forces combine in the baby’s mouth, and are most likely to be acting synergistically to remove milk in the most efficient way possible (the same is true of feeding by most dairy animals studied (sheep and goats) [15], and by pigs [16]).

Two of these forces are well illustrated in a recent study by Grassi et al. [17] who fitted two sensors to a pacifier, one monitoring positive pressure from the jaws and gums, and one measuring negative suction pressure within the oral cavity. Their figure (Fig. 1) shows the negative suction profile and positive pressure profile superimposed on each other, illustrating the relative scale and timing of these pressure forces at play during sucking on a pacifier.

Negative pressure (–150 to –200 mbar) is some 3–4 times greater in intensity than positive pressure (50 mbar). Negative suction pressure starts being generated while the jaws are relaxed/open, thereby ensuring the “teat” (or nipple/breast complex) is drawn into the oral cavity. Positive pressure by the jaws causes the gums to clamp on the base of the “teat,” thereby retaining it in the mouth. Shortly after jaw pressure reaches its peak, and starts to decline, negative pressure begins being regenerated. This is caused by lowering of the rear surface of the tongue, which, itself, is the end phase of the peristaltic wave as it completes its traverse of the oral cavity.

This is a key demonstration that even when feeding on an artificial teat (pacifier), the same natural forces are at play, despite them no longer having the normal function they would during breastfeeding.

The final force (7) is localized drawing down of the tongue surface adjacent to the nipple tip [7], the existence of which, and its role in milk removal, has only been confirmed in the past decade [11]. Unlike PTMs, this action is not obligate, but appears more facultative or opportunistic, only being superimposed on PTMs for a proportion of the time spent sucking. These localized depressions of the...
tongue surface are deployed at particular times during most feeds. Nonetheless, while they are ubiquitous, they cannot exist in isolation from PTMs; recent evidence (below) indicates they are generated by the same process. Their effect is to produce increased or added suction pressure local to the nipple surface; in all likelihood, this facilitates or enhances milk extraction. The phrase extractive tongue depressions (ETDs) will be used to refer to these, in view of their assumed function.

While the appearance of ETDs is difficult to predict, they are regularly associated with times of high milk outflow from the nipple. This association between peak negative suction pressure and “peak milk flow” was first revealed by Geddes et al. [11], who proposed that ETDs play a predominant role in milk extraction (in very much the same way as a breast pump would). The ability to detect “peak milk flow” was visually based on the movement of “echogenic flecks” in the space just beyond the nipple tip (stated to be “milk fat”). My personal belief is that these visual markers of milk flow are in fact evidence of “stable cavitation” [18], caused by microbubbles of carbon dioxide being drawn out of solution by the high negative suction pressure, usually being reabsorbed before the milk bolus is swallowed (this theory has yet to be tested or confirmed).

Engineering-Based Approaches to Modelling Milk Removal from the Breast

The evidence gleaned from multiple imaging studies has since been expanded by employing engineering-based models of the milk duct structure of the breast, and the baby’s sucking pattern, in order to generate theoretical data on milk flow, for comparison with real clinical data.

The first substantive attempt to develop a mathematical model of milk extraction during breastfeeding was undertaken by Zoppou et al. [19]. Drawing on knowledge current at the time, they compared the action of a breast pump, which used a cyclic pattern of suction, with that of a baby using both suction and expression. Their theoretical model caused them to conclude that there was an optimal time during the suck cycle to apply a compressive force, which increased milk flow over that produced by suction alone. Given their conclusion, it is somewhat surprising that recent models have not included a compressive component.

Two recent studies of milk removal from the breast have adopted an engineering-based approach [20, 21]. These studies have sought to create mathematical models which simulate the dynamic relationship between suction generated in the baby’s oral cavity, and the milk-filled duct system of the breast [20, 21]; both studies use data recorded directly during breastfeeding. They are elegant, complex, and sophisticated, although it can prove difficult to evaluate
them fully, in order to determine if any of the assumptions made might create erroneous conclusions. Modelling of the milk duct system of the breast is complex and will be bypassed in the discussion below, assuming them to be essentially accurate – that of Mortazavi et al. [21] is claimed to be more elaborate.

Elad et al. [20] have made some expansive claims about their work, but critical analysis of their study suggests some shortcomings. One noteworthy beneficial feature of their study is that their data analysis process allows them to use the hard palate as a register for movements of the tongue (Fig. 2). A relative weakness, in contrast, is that their data are derived from review of a relatively small selection of ultrasound images.

In brief, their methodology is as follows: 5–8 points are digitized on the hard palate (this is a manual process), to which a smooth curve is fitted by interpolation (red line in their Fig. 1A); the same process is applied to the tongue surface (green line). A set of tracings of the hard palate is collected for 4–6 suck cycles, which are then aligned with reference to the Hard-Soft Palate Junction, so that movement of the tongue relative to the hard palate can be visualized (identified as Fig 1A in their figure (Fig. 2A)).

Superimposed on this image (their Fig. 1C (Fig. 2A)) is a set of 28 equally spaced radiating lines (referred to as “polar coordinates,” numbered 1–27 in the figure), which radiate out from the scan head to above the hard and soft palate. The movement of the tongue surface is then plotted along every 5th or 6th polar coordinate, enabling the time lag, relative to the preceding focal coordinate to be visualized.

One apparent limitation of their approach is that 28 polar coordinates do not fully encompass the whole of the oral cavity. This might be regarded as a trivial issue, but the full passage of a suck across the oral cavity determines the overall suck duration, so that more lines would be required, up to at least 36, in order to embrace a full suckling action, including the prepharyngeal phase of swallowing.

Evaluating movement of the tongue surface relative to the hard palate, across four focal polar coordinates – #8, #13, #17, and #22 (illustrated), shows evidence of a phase shift between these separated lines (their Fig. 1E (Fig. 2B)).

In marked contrast, the time shift between ALL of the first 8 polar coordinates is evaluated and no phase shift is seen between individual lines. Based on their Fig. 1D (reproduced in my Fig. 2C), they assert that there is no phase shift between the lines, indicating that the “anterior tongue moves as a rigid body … ruling out the hypothesis of a peristaltic squeezing of the nipple” [20].

Personally, this line of argument appears misleading to me. Certainly, movement along the first three coordinates closest to the mandible (#1–3) is likely to be determined largely by up/down movement of the jaw, but beyond this point,
Tongue motility of a healthy infant during breast-feeding. (A) Submental US images with traced contours of the palate and the tongue’s upper surface in different frames of the movie clip (subject 24). (B) Contours of all the palate (red) and the tongue (green) from 150 frames of subject 24. (C) The contours of B after rigid registration around the rigid palate and the imposed polar coordinates. (D) Motility of the anterior tongue around anterior coordinates 1-8 after scaling (data from subject 41). The pattern fits the motility of a rigid body. (E) Motility of the posterior tongue around polar coordinates 8, 13, 17, and 22 after scaling (data from subject 41). The pattern fits the motility of a peristaltic wave. (F) Frequency distribution of the tongue contours around all of the polar coordinates. All regions of the tongue have the same dominant frequency of 1.56 Hz (i.e., 0.64 s per suckling cycle) (data from subject 41). HSPJ, hard-soft palate junction.

Fig. 2. A Figure taken from Elad et al. [20] – a full description is contained in the text. B, C After Elad et al. [10], highlighted enlargement of their their Fig. 1C–E.
there is evidence of propagation of a peristaltic wave from as early as polar coordinate #4, right through to #28.

The study by Monaci and Woolridge [22] merits discussion in this context, as it adopted a similar approach, but used signal processing techniques to analyze ultrasound records in real-time, thereby generating fully objective, automated results. They arbitrarily divided the oral cavity into three, spatially separated, non-overlapping sectors, equating to: (1) the anterior sector of the oral cavity, including nipple and front part of tongue (excluding lower jaw); (2) the middle sector of the oral cavity, comprising the mid-surface of the tongue and the space at the tip of the nipple in which milk accumulates; and (3) the posterior sector, comprising the oropharynx, where swallowing can be detected (Fig. 3).

These authors also examined the time shift between movements in each of these three areas. Ultrasound recordings from 29 mother/baby pairs (46 complete breastfeeds) were analyzed, although analysis was restricted to those periods when active sucking was taking place. Nonetheless, over 1 million frames of active sucking were analyzed in real-time by this technique.

If movement occurred in sector 1 first, a negative phase shift was recorded relative to sector 2; a zero phase shift indicated an absence of a phase shift between sectors 1 and 2; while a positive phase shift indicated movement in sector 2 preceded that in sector 1. In practice, this was caused by the movement in sector 2 being of larger amplitude than that in sector 1 and was commonly evidence for the presence of an ETD being inserted (i.e., an “added” suction element being superimposed on a peristaltic wave).

Figure 4 encompasses approximately 70 sucks, and illustrates the transition from a period of almost pure “peristaltic” sucking (frames 0–1,000), to a “vacuum” phase where ETDs predominate (alongside PTMs) (frames 1,000–1,500).
The “movement detection rectangles” were manually drawn, so needed to be redrawn when there was movement artefact. Despite this limitation, the signal processing approach was applied to all 46 breastfeeding episodes, totaling 16 h of recording. Overall results of the analysis are shown in Table 1.¹

PTMs were present throughout active sucking (100%), being: highly conspicuous for 78% of feeding, and predominating for over half of the time spent feeding, to the exclusion of ETDs (“suction/vacuum”). For a substantial period of feeding (27.5%), both PTMs and ETDS were equally visible, with no one

¹ Some caution is needed when generalizing from these data as they are based on a preliminary screening of ultrasound recordings; while some sections of feeding were not able to be analyzed, because of movement artefact, we regard them as substantively correct.
method predominating over the other. For 22% of feeding, the added suction elements (ETDs) appeared to predominate. This analysis shows that ETDs \([7, 12]\) were observable for roughly half of the time spent feeding.

The same authors used a second analytical technique involving automated mapping of the contour of the surface of the tongue. This technique is capable of showing the progression, across successive frames, of a peristaltic wave from the anterior to posterior of the oral cavity. In Figure 5, the peristaltic wave is seen rising in amplitude, then declining, as it transitions (left to right) from the front to the back of the oral cavity.

One final piece of evidence supplied by this latter technique is that when an ETD is generated, the space created is generated as part of the standard peristaltic wave, as it progresses across the zone where the ETD appears; it is both opened at its leading edge initially, then closed off again from its anterior edge (Fig. 6).

The two pictures show the contour of the dorsum of the tongue, which is automatically tracked (using the purpose-built software); the tongue outline is compressed left to right in this figure. The dotted line shows the tongue’s outline in the current frame, while the continuous line shows that in the previous frame. The circle circumscribes the mid-section of the baby’s tongue where the ETD is generated.

The upper picture shows the precise moment the ETD starts to be generated, as the continuous line shows an absence of any indentation, while the dotted line peels away markedly to create an indentation (marked with an X), representing the start of the formation of an ETD “pocket.” In the lower picture, just four

\[\text{Fig. 5. This figure shows the progression of a peristaltic wave from left (anterior) to right (posterior), across nine consecutive frames.}\]
frames later, the ETD “pocket” is clear in the continuous line, and it is just starting to be closed off again, from the front (marked with a Y). This is the clearest evidence to date that added suction elements (ETDs) are created by the same core peristaltic process.

Returning to the most recent engineering-based study, Mortazavi et al. [21] created a complex model of the milk duct system of the breast, which they then combined with directly measured suction pressure data (Fig. 7) (from several babies), to define the parameter boundaries of the mathematical model. Modelled milk output was then compared with clinical data on milk transfer for a single baby.
No data were collected on positive stripping pressure, so axiomatically, any such element was excluded from the model, despite it being an explicit component of one of the key studies they cited [19]. Any theoretical model which only assumes that the baby behaves like a mechanical suction pump is likely either to verify that presumption [20], or find that it is inadequate to explain clinical data on milk transfer [21].

In order to use sucking data in their model as parameter boundaries, sucking profiles were transformed into single harmonic, sinusoidal waveforms, seemingly all with a periodic frequency approaching 1 Hz (1 suck/s) (Fig. 8). The need to simplify natural data for incorporation into their model was no doubt necessary, but this constrains the baby’s sucking pattern to even more closely resemble an electric breast pump.

Their theoretical model simulated milk transfer by one baby, which was then compared with clinical data on intake by that baby. Based on this, the authors were forced to conclude that either sucking pressure alone, or total feed duration, did not account for: (a) the volume of milk removed, (b) the flow rate per unit time, or (c) the flow rate per suck.

This finding is unsurprising as it agrees with that of an earlier detailed study of the parameters of sucking pressure during breastfeeding [23], which was unable to find any association between suction and the 58% difference in intake between the first and second breast. Additionally, several authors have shown an inverse relationship between sucking pressure and milk flow during bottle-
Fig. 8. A selection of pressure profiles enlarged from Figure 7. The rate of nutritive sucking for all profiles is 0.92 sucks/s; the non-nutritive rate is not statistically different at 0.95 sucks/s.
feeds: the greater the resistance to milk flow caused by teat hole size, the greater the pressure exerted by the baby to remove milk [15].

A range of other factors are believed to explain the difference in milk intake between the first and second breast, including the “mother’s physiological response to sucking” [21], although no consideration appears to have been given to the fact that satiation by the baby can most commonly be observed when feeding from the second breast [24], and/or that less milk is available from the second breast as a result of the tendency to start breastfeeding on the breast offered last at the previous feed.

Both of the engineering-based theoretical models discussed above [20, 21] projected milk flow to be 1.85–3 times greater than measured intake by the baby, despite using many fewer branching ductal milk lobes than are naturally found in the lactating breast (the 5-lobe model [21] produced less of a discrepancy than the 2-lobe model [20]). Seeking to explain why milk flow was slower in reality, Mortazavi et al. [21] concluded that resistance to milk flow was greater than predicted in their model. They concluded this was likely to have been caused by factors including: the “deformed region of the areola-nipple,” a “reduction of ducts cross-sectional area,” and/or “elasticity of the tissue.” More generally, they alluded to other parameters, which included: “suckling” (mouthling or chewing movements were otherwise ignored), swallowing, and “breathing interruptions” (coordinating swallows with breathing may retard the rate of milk acquisition).

Validity of the Engineering-Based Mathematical Models

It is axiomatic that a mathematical model is only as strong as the number of assumptions on which it is based. Based on the methods reported in the development of these models, it is possible to identify several false assumptions which have been made, and incorporated into the models.

Addressing the study by Elad et al. [20] first, one of their key conclusions (from the technique shown in Fig. 2A–C) is that milk removal from the breast is caused by the rigid up/down movement of the baby’s jaws (impacting on the base of the nipple), combined with intraoral negative suction pressure. As a consequence, their model is based solely on the action of negative suction pressure, without any consideration of the possible role for the positive pressure wave created by the dorsum of the baby’s tongue. It should therefore come as no surprise that their model predicts that negative suction pressure alone can fully account for milk removal from the breast; essentially, theirs is more a kinetic model of how a mechanical breast pump works.
A key quality issue, likely to affect the validity of modelling studies, is the size of the sample on which any model is based. In the study by Elad et al. [20], 9 subjects, varying in age from 11 to 150 days were included, with a maximum of 15 s of sucking recorded. From these records, “four to six sucking cycles (i.e., about 150 frames) were selected for the analysis of tongue motion.” On the basis that six suck cycles last approximately 6 s, this signifies that data from a total of 54 s of feeding were used to generate the mathematical model. This may be a reason why Elad et al. [20] did not detect, or include in their analysis, the type of tongue movement described by Geddes and colleagues [11–14].

Certain assumptions may also be made to make a model less mathematically complex to compute. Mortazavi et al. [21] state that, in their study, the milk ducts are “assumed to be rigid.” An unstated corollary to this will be that the duct openings are also assumed to be rigid, being held open (patent) throughout the feed. This is recognized as a false assumption, as, in practice, the milk ducts are highly flexible and collapsible; it would not be easy to predict how they would behave dynamically under the combination of both positive pressure from the tongue and negative suction pressure from the oral cavity. Their model also assumes that negative suction pressure plays the sole role in milk removal. Perhaps as a consequence of this, a comparison of simulated data with clinical data from the same baby forces them to conclude that “suction pressure alone cannot account for milk removal from the breast” (the probable factors were discussed above).

**Key Physiological Features Not Included in Models**

A major physiological fact is overlooked by both these models, however, which is that the baby’s jaws repeatedly compress the nipple-breast/teat complex at its base, at the start of the suck cycle, and do so cyclically throughout the feed. This pressure (approx. 37.5 mm Hg) is likely to occlude the milk ducts with each suck, so it is not appropriate to assume that milk is drawn directly from the breast into the baby’s mouth, on the assumption that the milk ducts remain patent throughout. The milk-filled duct system of the mother’s breast represents a pressure gradient, confluent with the baby’s mouth, which is active at the onset of feeding. Hypothetically, this pressure gradient could ensure continuous movement of milk from within the breast into the baby’s mouth. At the start of the suck cycle, however, with closure of the baby’s jaws, it is no longer active and will only become active again at the end of the suck cycle, when the baby’s jaws reopen, and the teat ducts refill with milk from the breast.
This jaw closure, which causes the pressure gradient to be de-activated, persists for 75–80% of the suck cycle; so the pressure gradient cannot be characterized as being active throughout feeding. This is perhaps the biggest limitation of the two engineering-based models published to date, making them approximate much better to how a mechanical breast pump works. Neither provides a satisfactory theoretical explanation of how breastfeeding works (or of manual breast expression for that matter). Further concern should be raised over the assumption that the milk duct apertures remain open throughout; this is unlikely given the close approximation of the nipple to the soft tissues of the baby’s mouth, and the high suction pressures generated within the oral cavity.

**Which Force Is Primary in Causing Milk Removal from the Breast?**

As suggested above, the *active pressure gradient* could make it possible for milk to be delivered continuously from the breast to the baby’s mouth, were it not for the “gating” effect of the baby’s jaws. Accordingly, the milk available on each suck is limited to that captured in the milk ducts lying within the baby’s mouth; milk cannot be extracted directly from the breast. A further fact, which should not be overlooked, is that the milk duct openings are very much narrower (by up to 50 times) than the dilated milk-filled ducts leading to them. So, an essential corollary to the question above is: “What force is responsible for opening the duct ends?”

Based on the proposition of Geddes and colleagues [10–14], can it be the case that *localised added suction* (ETD) at the nipple surface is the force responsible? The answer is likely to be an emphatic “No.” Any level of suction pressure applied outside the nipple surface (if this exceeds the positive milk pressure created by the mother’s MER), is likely to cause collapse of the teat openings. While suction can be transmitted through a fixed aperture, and propagated back along a rigid tube, this cannot occur in the flexible, collapsible milk duct system of the breast. Nipple duct opening, therefore, cannot be achieved from outside the nipple surface.

Instead, this can only be achieved from within, by increasing *intra-ductal pressure*. This is precisely what the peristaltic tongue movements do. Having captured milk within the milk ducts held in the oral cavity, the peristaltic wave of compression squeezes this milk towards the nipple end; the resulting rise in intra-ductal pressure forces the milk duct ends open. Only when this has happened, might *extra-ductal pressure* (added intra-oral suction from an ETD) be capable of enhancing either the rate of milk extraction, or the net volume of milk transferred during that suck. The mechanism by which added suction is likely
to achieve this is by extending the suck duration, potentially achieving more effective emptying of the ducts.

From this perspective, not only are peristaltic tongue movements (PTMs) the obligate, primary tongue movement, present throughout active sucking, they also appear to be the primary mechanism by which milk is forced towards the duct openings, and out into the baby’s mouth. It may be deduced from this that the efficiency of such a mechanism will depend on the surface area of the nipple-breast “teat” complex lying against the baby’s tongue. In addition, the wider the baby’s mouth is flanged, the better will be its apposition to the breast; resulting in a greater mouthful of breast tissue being taken by the baby. Both these key features will be enhanced by maximising the “positioning” and “attachment” of the baby at the breast.

A Final Piece of Evidence

One final piece of unique evidence comes from a historical study, not from any recent research. In the 1980s, Alan Lucas, then based at the John Radcliffe Hospital in Oxford, came up with the novel idea of measuring milk transfer from a mother to her baby directly, by placing a flow transducer between them, housed in the tip of a latex nipple shield [25]. The research team (Bio-Engineering Unit) developed a Doppler ultrasound flow transducer which insonated an area of parallel milk flow, created as breast milk passed through the transducer body. This technique provides completely unique views of instantaneous milk transfer during suckling [26]; I have been able to revisit a proportion of the original milk flow traces, undertaking some fresh analysis of them, in an attempt to resolve some the issues emerging from the “revised suckling physiology” above.

If, as Geddes et al. [11] assert, added suction (ETDs) is the predominant force in milk removal from the breast, then one would be likely to observe a “mid-suck” peak in milk flow, with relatively little milk flow either side of this. In practice, this is not the case – peak milk flow is invariably seen early in the suck cycle (first 20%), tailing off towards the end (Fig. 9). In many sucks, following an early high amplitude flow, a later more attenuated phase of irregular milk flow may be observed; this is most conspicuous in sucks of longer duration. These sucks are most likely to be those which include an ET, which were shown by Eishima [7] to extend the suck duration.

More commonly, the two phases of milk flow grade into each other, so first we have a high-amplitude, short-duration flow, followed by a lower-amplitude, longer-duration flow. The net contribution each of these makes to milk transfer may be the same, although it is important to remember that the sec-
ondary peak of milk flow may be absent in a large proportion of sucks. This novel source of information about milk flow during suckling suggests that baseline suction and PTMs are uniquely responsible for initiating and maintaining milk flow on each and every suck. When ETDs are superimposed on the incipient rhythm, they appear to enhance milk flow, mainly by sustaining it over a longer duration.

This unique insight into the process of milk transfer has been provided, not by new engineering-based models, but by a much earlier piece of research. Nonetheless, we have only recently been able to explain fully the complex shape of the milk flow profile in light of the evidence that both PTMs and ETDs coexist during breastfeeding, demonstrating that the baby both suckles and sucks milk from the breast.

**Clinical Implications of the “Revised Suckling Physiology”**

Based on the evidence presented above, it is reassuring to learn that the standard tenets of good breastfeeding technique remain as true today as when they were first proposed [27, 28]. Enhancing the Positioning and Attachment of the baby at the breast will have the specific benefit of maximizing mouth:breast apposition. The greater the mouthful of breast tissue the baby takes, the further it will extend into the oral cavity, so providing a greater opportunity for the dorsum of the baby’s tongue to compress the underside of the teat. Explicitly, this will maximize the baby’s ability to amplify intraductal pressure during the suck, thereby boosting milk expression. Only when the duct ends have been opened by pressure from within, will added suction (ETDs) be capable of enhancing milk flow.
No intervention has yet been identified which allows the level of suction that the baby produces to be modified (although the faster the rate of milk flow, the less suction pressure is likely to be applied). Accordingly, the basic tenets of Positioning and Attachment would apply here also.

Disclosure Statement

M.W.W. was remunerated for his participation in this workshop; his honorarium offsetting, in part, the time invested in preparation of this paper. The views expressed within it are entirely his own, and do not reflect those of any commercial sponsor.

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Alan Lucas took an overview of the scientific evidence that supports breastfeeding. He identified that three key pillars of breastfeeding science were underpinned by flawed science and thinking. That breastfeeding has evolved over millions of years of mammalian evolution, spawned the view human breast milk must be the perfect diet for human infants. But this view does not take into account the competing needs of mother and offspring, nor the current mismatch between our ancient genes and modern environment (evolutionary discordance) which may result in disease. He noted that 1,500 papers on the composition breast milk were too flawed to derive realistic values for the dietary intake of breastfed babies. The use of such data by formula manufactures resulted in overfeeding of babies contributing to the epidemic of obesity and cardiovascular disease risk in later life. And he noted that the numerous proposed beneficial outcomes of breastfeeding were mostly based on potentially confounded observational studies. He addressed these issues by showing that more modern research and understanding was resolving the flaws of past work and thinking. This newer work puts breastfeeding medicine on a sounder scientific footing – and this is in the interests of improving the care of infants and the health outcomes of populations.

Mike Woolridge delved deeply into the mechanical aspects of suckling. He noted two long-standing principles about the way babies removed milk from the breast – one mechanism is suction, and the other, the wave-like movements of the tongue that exert pressure on the undersurface of the nipple and breast in
the oral cavity. These aspects were confirmed by imaging studies from the 1950s and underpinned successful “best practice” in breastfeeding management. He explained however that more recently the process of suckling has been revisited with more sophisticated imaging techniques and the application of engineering approaches to build new mathematically complex models to explain how breastfeeding works. He critically appraised the strengths and weaknesses of the new research but gratifyingly concluded that a new consensus on the principles of suckling, whilst extending our understanding, does not in any way undermine or change well-tried and effective best practices for the management of breastfeeding – indeed more strongly reaffirms them. Such practices impact on the success of suckling, and thus in turn help underpin the efficacy of breastfeeding as a public health intervention.

Alan Lucas
Physiological Effects of Feeding Infants and Young Children Formula Supplemented with Milk Fat Globule Membranes

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Abstract
Dietary supplementation with bovine milk fat globule membrane (MFGM) concentrates has recently emerged as a possible means to improve the health of infants and young children. Formula-fed infants are of special interest since infant formulas traditionally have lower concentrations of biologically active MFGM components than human milk. We identified 6 double-blind randomized controlled trials (DBRCT) exploring the effects of supplementing the diet of infants and children with bovine MFGM concentrates. Two studies found a positive effect on cognitive development in formula-fed infants. Three studies found a protective effect against infections at different ages during infancy and early childhood. We conclude that supplementation with MFGM during infancy and childhood appears safe, and the studies indicate positive effects on both neurodevelopment and defense against infections, especially in formula-fed infants. However, due to the small number of studies and the heterogeneity of interventions and outcomes, more high-quality DBRCTs are needed before firm conclusions can be drawn on the likely health benefits of MFGM supplementation to infants and children.

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Introduction

An increasing number of studies have reported various health benefits from oral supplementation with bovine milk fat globule membrane (MFGM) to humans of different ages, including infants and children [1, 2]. The MFGM is formed during the release of milk fat from the endothelial cell of the lactating mammary gland and is composed of a phospholipid and cholesterol triple layer which contains proteins and glycoproteins [3] (Fig. 1). Milk phospholipids, sphingomyelins, and gangliosides are largely located on the MFGM, although phospholipids are also secreted as smaller vesicles devoid of a triglyceride core, which typically separate from the whey fraction [3, 4]. The proteome of the human MFGM is very complex with several hundred proteins identified, including mucins, butyrophilin, lactoferrin, and lactadherin [5, 6]. Bovine MFGM-rich fractions contain approximately the same number of proteins [7]. MFGM is also rich in sialic acid as part of gangliosides [4] and glycosylated proteins. The genes regulating MFGM synthesis are conserved across species suggesting a functional benefit of this fraction in milk [8], even if the detailed MFGM composition varies among species [6].

Breastfed infants have a higher intake of MFGM components than formula-fed infants because, traditionally, the MFGM fraction is discarded with the milk fat which is replaced by blends of vegetable oils as the source of fat in infant formulas. Resulting from advances in dairy technology, bovine MFGM concentrates are now commercially available and possible to use as a supplement to foods, including infant formulas.

Physiological Effects of Single Components of the Milk Fat Globule Membrane

Dietary gangliosides [9], sialic acid [10], and sphingomyelin [11] have been shown to be important for optimal brain development and function in different animal models. However, it should be noted that some of these models are disease models or models with inhibited de novo synthesis, which is far from supplementing a healthy infant or child. In a small study on premature infants with a birth weight <1,500 g, infants receiving formula with high sphingomyelin content (20 vs. 13% of all phospholipids in milk) to cover shortages of breast milk performed better than those fed the lower content at neurobehavioral follow-up between 6 and 18 months corrected age [12]. Further, oral sphingomyelin [13], as well as a bovine MFGM concentrate [14], increased maturation of the intestine in rats. Gangliosides have also been suggested to play an important role in the development of intestinal microbiota composition, gut immunity, and, consequently, in the de-
Effects of Feeding Infants and Young Children MFGM

Other components of MFGM are also involved in the defense against infections, e.g., the glycoproteins butyrophilin, lactadherin, and mucins [16], which all have antimicrobial effects, and the lipid fraction of bovine MFGM has antiviral effects in vitro [17]. Both lipid and protein components of MFGM have anticancer effects in vitro [18], and intake of MFGM in early life has also been suggested to protect against obesity later in life [19].

Clinical Studies on Milk Fat Globule Membrane Concentrates Fed to Infants and Children

In a literature search (August 31, 2017), we identified 6 double-blind randomized controlled trials (DBRCT) exploring the effects of supplementing the diet of infants or children with MFGM (Table 1):
In a Peruvian DBRCT, 550 healthy, primarily breastfed 6- to 11-month-old infants consumed 40 g/day of an instant complementary food fortified with 1 recommended dietary allowance of multiple micronutrients and a protein source for 6 months. They were randomized to the protein source being either an MFGM-enriched protein fraction (Lacprodan® MFGM-10; Arla Foods Ingredients) or skim milk powder (control group) [20]. There was no difference between the groups in the incidence of diarrhea, but longitudinal prevalence of diarrhea was significantly lower in the MFGM group compared to the control group (3.84 vs. 4.37%, \( p < 0.05 \)). In a multivariate model adjusted for initial anemia and potable water facilities, the incidence of bloody diarrhea was lower in the MFGM group, with an adjusted OR of 0.59 (95% CI 0.34–1.02, \( p = 0.025 \)).

In a DBRCT performed in Indonesia, 70 term infants were randomized to a control formula or an infant formula enriched with bovine milk gangliosides, provided as a complex bovine milk lipid fraction (Annum Infacare; Fonterra Cooperative Group, Auckland, New Zealand) [21]. A breastfed reference group (BFR) (\( n = 40 \)) was also recruited. The intervention started between 2 and 8 weeks and continued until 24 weeks of age. After adjustment for socioeconomic background variables, the hand-eye coordination IQ (129.5 vs. 122.0, \( p = 0.006 \)), performance IQ (131.1 vs. 123.2, \( p < 0.001 \)), and general IQ (125.4 vs. 120.6, \( p = 0.041 \)) measured with the Griffiths Mental Developmental Scale were

**Table 1.** Double-blind randomized controlled trials exploring the effects of milk fat globule membrane (MFGM) supplementation to the diet of infants or children

<table>
<thead>
<tr>
<th>Study</th>
<th>Age Supplementation</th>
<th>Main results for the MFGM group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zavaleta et al. [20]</td>
<td>6–11 months MFGM (Lacprodan® MFGM-10; Arla Foods Ingredients)</td>
<td>Lower longitudinal prevalence of diarrhea                                                        Lower incidence of bloody diarrhea</td>
</tr>
<tr>
<td>Gurnida et al. [21]</td>
<td>2–8 to 24 weeks Complex milk lipids (Annum Infacare; Fonterra Cooperative Group)</td>
<td>Higher hand-eye coordination IQ, performance IQ, and general IQ</td>
</tr>
<tr>
<td>Veereman-Wauters et al. [22]</td>
<td>2.5–6 years, during 4 months MFGM (INPULSE®; Büllinger SA)</td>
<td>Fewer days with fever and lower parental scoring of internal, external, and total behavioral problems</td>
</tr>
<tr>
<td>Poppitt et al. [23]</td>
<td>8–24 months, for 12 weeks Complex milk lipids (Fonterra Cooperative Ltd.)</td>
<td>No difference between groups</td>
</tr>
<tr>
<td>Timby et al. [24, 26–28]</td>
<td>&lt;2 to 6 months MFGM (Lacprodan® MFGM-10; Arla Foods Ingredients)</td>
<td>Higher cognitive score                                                                            Higher incidence of otitis media Higher serum cholesterol</td>
</tr>
<tr>
<td>Billeaud et al. [29]</td>
<td>14 days to 4 months Lipid-rich MFGM fraction (Fonterra Cooperative Group)</td>
<td>Weight gain was noninferior                                                                      Higher rate of eczema in the protein-rich MFGM group</td>
</tr>
</tbody>
</table>
higher in the ganglioside-supplemented group than in the control group, and the ganglioside-supplemented group did not differ from the BFR group.

In a Belgian DBRCT, 253 preschool children aged 2.5–6 years received 200 mL of a chocolate formula milk daily for 4 months [22]. They were randomized to a formula without phospholipids (placebo group) or enriched with 500 mg of phospholipids with the addition of 2.5% of a phospholipid-rich MFGM concentrate (Inpulse; Büllinger SA, Büllingen, Belgium) (intervention group). The intervention group had fewer days with fever (mean ± SD: 1.71 ± 2.47 vs. 2.60 ± 3.06, \( p = 0.028 \)), and lower parental scoring of internal (\( p < 0.003 \)), external (\( p < 0.005 \)), and total (\( p < 0.002 \)) behavioral problems measured by the Achenbach System of Empirically Based Assessment (ASEBA). However, ASEBA scoring was only performed after the intervention but not at baseline, and differences were not confirmed when the children’s teachers made the scoring.

In an Indian DBRCT, 450 infants between 8 and 24 months of age were randomized to a daily dose of milk powder supplemented with 2 g of a spray-dried ganglioside concentrate (Fonterra Cooperative Ltd.) or milk powder only (control group) for 12 weeks [23]. There was no difference between the groups, nor in the primary outcome rotavirus diarrhea, or in secondary outcomes including all-cause diarrhea. However, the authors noted that the incidence of rotavirus diarrhea during the study period was lower than expected, making the study under-powered as compared to the intention of the design.

In a Swedish DBRCT, 160 formula-fed healthy term infants were randomized to receive an experimental formula (EF) supplemented with a protein-rich MFGM fraction (Lacprodan® MFGM-10; Arla Foods Ingredients) or standard formula (SF) from <2 to 6 months of age. The EF had lower energy (60 vs. 66 kcal/100 mL) and protein (1.20 vs. 1.27 g/100 mL) densities, and MFGM-proteins made up 4% (wt/wt) of the total protein content in the formula. In addition, a BFR group including 80 infants was also studied. The formula-fed infants regulated their ingested volumes by increasing meal size, resulting in no differences in energy intake, protein intake, blood urea nitrogen (BUN), serum insulin level, or growth, including body fat percentage, up to 12 months of age [24]. The surprisingly high level of self-regulation for the bottle-fed infants might be explained by a low level of parental control in the study population [25].

At 12 months of age, the EF group achieved higher scores (mean ± SD) in the cognitive domain of Bayley III (105.8 ± 9.2) than the SF group (101.8 ± 8.0, \( p = 0.008 \)) and did not differ from the BFR group (106.4 ± 9.5, \( p = 0.73 \)) [24]. During the intervention, the EF group had a lower incidence of acute otitis media than the SF group (1 vs. 9%, \( p = 0.034 \)), a lower incidence and longitudinal prevalence
of antipyretic use, lower concentrations of serum IgG against pneumococci after vaccination and a lower prevalence of *Moraxella catarrhalis* in the oral microbiota, all suggesting an infection-protective effect of EF [26, 27]. During the intervention, the EF group gradually reached higher serum cholesterol concentrations than the SF group, and there was no significant difference between the EF and BFR group at 6 months of age [28].

In a multicenter noninferiority DBRCT, 199 healthy term infants were randomized to 3 different formulas from 14 days to 4 months of age; a SF (control), a formula enriched with lipids (MFGM-L; Fonterra Cooperative Group Ltd), and a formula with a protein-rich (MFGM-P, Lacprodan® MFGM-10, Arla Foods Ingredients) bovine MFGM fraction, respectively [29]. Weight gain was noninferior in the MFGM-L and MFGM-P groups compared with the control group. Adverse events and morbidity rates were similar across groups except for a higher rate of eczema in the MFGM-P group (13.9 vs. 1.4% in the MFGM-L group and 3.5% in the control group, *p* = 0.001). It is, however, not clear how and when eczema was diagnosed, and the number of infants diagnosed were few in the MFGM-L and control groups (1 and 2, respectively). The authors also concluded that care must be taken in interpreting the exploratory endpoints. A higher risk of skin rash was not confirmed in a Swedish study [30] which studied the same MFGM-P fraction.

**Conclusions**

Studies on the supplementation of bovine MFGM to the diet of infants and children have shown promising results regarding both neurodevelopment and defense against infections. These findings are supported by known effects of individual components of MFGM mostly based on in vitro and/or animal studies. However, the scientific base of knowledge for MFGM supplementation to infants and children is still limited. The number of published studies on MFGM supplementation to infants and children is small, and the interventions are heterogeneous: different MFGM concentrates have been given for different durations at different ages and with different main outcomes. However, MFGM supplementation seems safe down to the age of the first week of life in term infants, as no serious adverse effects have been reported.

Infant formulas supplemented with bovine MFGM concentrates have already been launched on many markets, but before any general recommendations or guidelines of MFGM use in infants and children can be given, more high-quality DBRCTs are needed.
Disclosure Statement

O.H. has participated as a clinical investigator and/or scientific advisory board member, speaker, and consultant for Semper, Hero, Mead Johnson Nutrition, Arla Foods, Arla Foods Ingredients, Nestlé Nutrition Institute, and Hipp. M.D. has participated as clinical investigator and/or speaker for Hero, Semper, Baxter, Nutricia, and Nestlé Nutrition Institute. T.G. has participated as clinical investigator/speaker for Semper and Hero. B.L. has participated as a clinical investigator and/or scientific advisory board member, consultant, and speaker for Semper, Hero, Mead Johnson Nutrition, Arla Foods, Arla Foods Ingredients, Albion, Humana, Biostime, and Nestlé Nutrition Institute. N.T. has participated as a clinical investigator and/or speaker for Hero and Semper.

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Human Milk Oligosaccharides: Factors Affecting Their Composition and Their Physiological Significance

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Abstract

Human milk oligosaccharides (HMOs) are elongations of the milk sugar lactose by galactose, N-acetylg glucosamine, fucose; and sialic acid. The HMO composition of breast milk is strongly influenced by polymorphisms of the maternal fucosyltransferases, FUT2 and FUT3, and by the stage of lactation. Clinical observational studies with breastfed infant-mother dyads associate specific HMOs with infant gut microbiota, morbidity, infectious diarrhea, and allergies. Observational and basic research data suggest that HMOs influence the establishment of early-life microbiota and mucosal immunity and inhibit pathogens, thereby contributing to protection from infections. Clinical intervention trials with infant formula supplemented with the single HMO, 2′-fucosyllactose (2′FL), or with 2 HMOs, 2′FL and lacto-N-neotetraose (LNnT), demonstrated that they allow for age-appropriate growth and are well tolerated. A priori defined exploratory outcomes related feeding an infant formula with 2 HMOs to fewer reported illnesses of the lower respiratory tract and reduced need for antibiotics during the first year of life compared to feeding a control formula. In parallel, early-life microbiota composition shifted towards that of breastfed infants. Together, HMOs likely contribute to immune protection in part through their effect on early-life gut microbiota, findings that warrant further clinical research to improve our understanding of HMO biology and significance for infant nutrition.

Introduction

What are human milk oligosaccharides (HMOs)? What is their importance for infant nutrition? These questions have intrigued scientists and pediatricians alike for over a century. Advances in analytics as well as large-scale synthesis
technologies stimulated great progress in recent years. These provided the materials and tools that enabled the detailed and accurate measurement of HMO quality and quantity, and the study of HMOs in basic research models, and through clinical observational studies and intervention trials.

“HMOs are not HMOs,” meaning that one specific HMO is not equal to another HMO, especially when considering structure-function relationships. Chemically, HMOs are elongations of the milk-specific sugar lactose in different linkages by one or a combination of the following monosaccharides: L-fucose (Fuc), D-galactose (Gal), N-acetyl-D-glucosamine (GlcNAc), and N-acetylneuraminic acid (sialic acid). Gal and GlcNAc generally elongate lactose as a disaccharide Gal-GlcNAc. The numerous and diverse HMOs produced might be categorized by specific structural features brought about by different glycosyltransferases involved in their synthesis. However, many HMOs combine different structural features.

Breast milk, the recommended and naturally adapted nutrition for infants, is associated with a reduced risk for infection-related illnesses and possibly for diabetes and overweight, while the situation for allergies is less clear [1]. This suggests that breast-milk-specific components such as HMOs and other bioactives may contribute to such benefits.

Due to their structural similarity with mucosal glycans and their nondigestible nature, HMOs expectedly affect numerous glycan-mediated processes like the colonization of the early-life microbiota and the infectivity of pathogens (Fig. 1a). Based on clinical observational and basic research data, HMOs act in a structure-function-specific way helping the (i) establishment of a mucous-adapted microbiome, (ii) resistance to pathogens, and (iii) reactivity of the mucosal barrier and immunity, thereby contributing to immune protection.

Here, we briefly review genetic and environmental factors affecting HMO composition in breast milk and the physiological role of HMOs as supported by clinical observation studies, preclinical research on mode of action, and insights from clinical intervention trials.

Maternal Glycosyltransferase Polymorphisms Affect HMO Composition

HMOs resemble the blood group antigens and further sialylated glycans that cover the human mucosa. The same glycosyltransferases are generally involved in the synthesis of mucosal cell glycans and mammary gland-expressed HMOs. The fucosyltransferases FUT2 (secretor gene) and FUT3 (Lewis gene) are the best described due to their natural polymorphisms in humans [2] (Fig. 2a). Spe-
specific genetic polymorphisms abolish their respective enzyme activity. Thus, specific HMO structures that depend on FUT2 or FUT3 can be identified. FUT2-dependent HMOs all contain α1,2-linked fucose, for example 2′-fucosyllactose (2′FL), lactodifucosyllactose (LDFT), and lacto-N-fucosylpentose (LNFP)-I. Interestingly, trace amounts of 2′FL were found in breast milk of presumed FUT2-
Fig. 2. Human milk oligosaccharide (HMO) composition 3 months postpartum by FUT2 and FUT3 status with schematic illustration of typical HMO in one or the other group (a). HMO dynamics at different stages of lactation (replotted from Austin et al. [3]) depicting mean concentrations with standard deviations (b).
negative mothers in Asian populations [3, 4], indicating that the nature of these inactivating polymorphisms and thus the HMO profile may be population specific [5]. Typical FUT3-dependent HMOs are LNFP-II, lacto-N-difucosylhexose (LNDFH)-I containing α1,4-linked fucose, and to a lesser extent 3-fucosyllactose (3FL) and LDFT containing α1,3-linked fucose. In breast milk that does not contain any detectable LNFP-II, reduced amounts of HMOs with α1,3-linked fucose on glucose and increased amounts of those with an α1,3-linked Fuc on GlcNAc are found. Hence, another FUT (e.g., FUT4, FUT5, FUT6, FUT7, or FUT9) is also involved in HMO formation with an α1,3-linked Fuc on GlcNAc and glucose.

The absence of a functional FUT2 or FUT2 and FUT3 affects the concentration of total HMOs in milk when expressed as the sum of all quantified HMOs [2] (Fig. 2). While some HMOs increase when FUT2 is missing (e.g., LNnT and 3FL), in the absence of fucosylation additional larger nonfucosylated HMOs might also be produced.

To date, no common genetic polymorphisms for sialylated HMOs have been described, indicating that if inactivating polymorphisms in sialyltransferase genes exist, they are extremely rare. From mouse studies, the sialyltransferases ST6Gal1 and ST3Gal4 are involved in the synthesis of 6′-sialyllactose (6′SL) and 3′-sialyllactose (3′SL), respectively, with a further sialyltransferase, probably ST3Gal1, also making 3′SL [6].

Another mechanism affecting HMO composition is probably the donor and acceptor substrate availability, as suggested by the increase in 3FL when the major fucosyl-HMO 2′FL decreases in concentration [3].

Interestingly, HMO concentrations change during the stage of lactation with different HMOs showing different dynamics [3]. HMOs like 6′SL or LNNnT decrease more rapidly during the first weeks of lactation, while 2′FL and 3′SL, for example, decrease more slowly over a longer time period, and again others, like 3FL, actually increase in concentration with time of lactation (Fig. 2b).

Such compositional changes due to the genetic background of mothers and stage of lactation can confound observations relating HMOs to clinical parameters in the breastfed infants and, therefore, need to be considered.

HMO Composition and Maternal Diet, Gestational Age, and Physiological State of the Infant

HMO concentrations in colostrum, transitional milk, and mature milk seem not to change between mothers giving birth to preterm (n = 18; gestational age <37 weeks) and term (n = 14; gestational age ≥37 weeks) infants [2]. Further,
Fucosylated and sialylated HMOs were reported to be similar between preterm and term milk, although preterm milk seemed more variable in the expression of fucosylated HMOs [7].

Today, we do not know whether and how maternal diet might influence HMO composition. A recent observational study including 33 breastfeeding mothers and their infants from the Gambia, Africa, reported a significantly higher HMO content in milk at 20 weeks of lactation in the dry season ($n = 21$) than the wet season ($n = 12$) [8]. The authors propose a possible link to the higher energy intake during the dry season. In 2 other African mother-infant cohorts from Malawi ($n = 88$ and $n = 215$), total HMOs and also sialyl- and fucosyl-HMOs were lower 6 months postpartum in the breast milk of mothers having severely stunted infants compared to those with normal-size infants [9]. These studies suggest that maternal nutritional and health status may affect HMO composition.

By analogy, higher maternal body mass index and gestational weight gain, which generally reflects an altered metabolic physiology, might affect HMO composition. Studies to this end are currently ongoing [10; Binia et al.: abstract at FASEB Science Research Conferences in 2017]. Suitable studies are warranted to investigate possible alterations in HMO composition due to maternal energy and specific nutrient intake.

The HMO Composition Is Associated with the Gut Microbiota in Infants

The early-life microbiome has a major impact on the developing immune system, itself being an important element by providing pathogen colonization resistance, for example. Interestingly, the establishing intestinal microbiota also contributes, via an innate lymphoid cell-mediated process, to improved protection against respiratory tract infection [11]. The pioneers of human milk and breastfeeding research observed a strong link between breastfeeding and immune protection to infectious morbidity and mortality. Breastfed infants were recognized to harbor an early gut microbiota dominated by bifidobacteria, not seen in formula-fed infants, and a human-milk-specific “bifidofactor” was identified in the HMO fraction of breast milk [12].

From research on early-life microbiota, we know that bifidobacteria can utilize and grow on different individual HMOs in a strain-specific way [13, 14]. Several studies observed an increased bacterial metabolic activity upon growth on HMOs, exemplified by the formation of the short-chain fatty acid acetate [15, 16]. Noteworthy, numerous potentially pathogenic bacteria from the Enterobacteriaceae group were shown not to grow on individual HMOs as the sole carbon source [17], while growth of other pathogens, like Streptococ-
**Human Milk Oligosaccharides** (group B Streptococcus, GBS) was shown to be inhibited by HMOs [18, 19].

Recently, LNnT in breast milk was associated with *Bifidobacterium longum* ssp. *infantis* abundance [8]. In bi-associated gnotobiotic mice harboring only 1 *Bacteroides* and 1 *B. longum* ssp. *infantis* strain, LNnT lead to bifidobacteria dominance although both bacteria could actually use LNnT in vitro [20]. In gnotobiotic mice humanized with 7 human microbes, *B. longum* ssp. *infantis* also showed higher abundance when these mice were fed 2'FL combined with LNnT as compared to LNnT alone [Sprenger et al., unpubl. observation], although *B. longum* ssp. *infantis* is able to grow on many different HMOs, including LNnT, as substrate [13].

Genomic and glycomic analyses in infants provided further evidence for a role of HMOs in shaping the early infant gut microbiome, revealing associations between individual HMOs and bacterial genera in infant stool [21–23]. A *Bifidobacterium*-dominated gut microbiota in breastfed infants (*n = 105*) at 4 months of age was associated with breast milk containing FUT2-HMOs [24]. The FUT2 status of the infants and its possible confounding effects on the infant microbiota profile were not assessed, despite earlier data proposing the FUT2 status itself can influence the gut microbiota at least in adults [25]. In another cohort, the analysis of a relatively small subgroup of infants exclusively breastfed for 4 months (*n = 14*) showed an association of maternal FUT2-positive status with higher *Bifidobacterium* abundance up to 2–3 years of age [26]. However, no statistically significant HMO effects on global *Bifidobacterium* shifts were reported in another recent study of 33 Gambian mothers and infants [8], while the abundance of individual bifidobacteria like *B. longum* ssp. *infantis* still correlated with LNnT concentrations in breast milk. These first reports reveal the need for larger observational studies of similar design, including comprehensive HMO analysis of breast milk and infant FUT2 phenotyping to gain a more robust understanding of the link between HMO and infant gut microbiome composition.

Today, clinical observations in conjunction with basic research data suggest that FUT2-HMOs, like 2'FL and LNFP-I, but likely also other non-FUT2-dependent HMOs, like LNnT for example, are involved in the establishment of a *Bifidobacterium*-dominated early-life gut microbiota. In vitro studies help to understand HMO-related microbial metabolic capacities and strain specificities, while animal and human observational studies indicate that the interaction between bacteria and the gut mucosa reflect a more complex picture. Hence, with infant health in mind, it is central to gain a better understanding of HMO effects on the microbiome dynamics in their natural ecosystem through a holistic and ecology-inspired approach.
HMO Composition Is Linked to Infection Risk in Infants

HMOs were studied in relation with infectious diarrhea incidence in a cohort of Mexican mothers and infants \( (n = 93) \) [27, 28]. Higher breast milk concentrations of \( \alpha_{1,2} \)-fucosylated HMOs were associated with a lower incidence of all-cause moderate-to-severe diarrhea. The most frequently identified cause of diarrhea in the cohort was *Campylobacter jejuni* followed by calicivirus and enteropathogenic *Escherichia coli*. Specifically, higher concentrations of 2′FL and LNFP-I in breast milk correlated with a lower incidence of *C. jejuni* and calicivirus diarrhea, respectively. These observations during the breastfeeding period did not persist in the period after breastfeeding, indicating a possible transient HMO effect in the protection from infectious diarrhea. This fits their presumed role as anti-adhesive antimicrobials. Experimental data from preclinical models also show protective effects of 2′FL from *C. jejuni* [29] and aggregating invasive *E. coli* [30]. From these data, 2′FL and other FUT2-HMOs seem to act as soluble ligands blocking *C. jejuni* from adhering to gut epithelial cells, while the protection from *E. coli* might rather be due to an anti-inflammatory effect, possibly combined with the modulation of the gut microbiota composition.

Glycans containing \( \alpha_{1,2} \)-linked Fuc expressed on epithelial cells of FUT2-positive infants could act as receptors for pathogen binding, conferring a risk to specific infectious diseases for this population [31]. Genetic studies have shown that infants and children with a nonfunctional FUT2 gene have strain-specific protection against norovirus and rotavirus [32, 33]. For specific rotavirus strains, susceptibility depends on FUT2 but also on FUT3 status [34]. Experimentally, infectivity of some rotavirus strains was reduced by the FUT2 HMO 2′FL, while other viral strains were affected by sialylated HMO, namely 3′SL and 6′SL [35]. Similarly, 2′FL also bound to specific norovirus strains [36].

Besides interfering with pathogen attachment to the host mucosa, HMOs were recently reported to exert bacterial-growth-inhibitory activities on pathogenic GBS [18, 19, 37], a major cause of sepsis in preterm infants. Growth of GBS was specifically inhibited by LNT and LNFP-I, while sialylated HMOs or galactooligosaccharides (GOS) had no effect [19]. Experimental data suggest a putative glycosyltransferase of GBS to be involved [19]. Possibly pointing to a similar mechanism, HMOs from milk of a FUT2-negative mother were shown to have bacteriostatic properties via an alteration in biofilm formation [18]. In an observation study of 183 Gambian infant-mother pairs, FUT3-positive mothers were reported to be less likely carriers of GBS, as were their infants at birth [37]. Interestingly, infants of FUT3-positive mothers were also more likely to clear GBS colonization from birth to 2–3 months of age compared to infants of FUT3-negative mothers.

In a pilot study of 49 mother-infant pairs, higher breast milk concentrations of the FUT3-HMO LNFP-II at 2 weeks were associated with a lower risk of re-
spiratory and gastrointestinal illnesses at 6 and 12 weeks in infants [38]. This association was no longer significant after the breastfeeding period. Similarly, in a nested case cohort study of 143 HIV-exposed uninfected children from Zambia, higher concentrations of fucosylated HMOs in breast milk 1 month postpartum related to a lower risk of mortality up to 2 years of age [39]. In another small mother-infant cohort from the Gambia \((n = 33)\), higher relative breast milk concentrations of fucosylated HMO (sum of LNFP-I and LNFP-III) and concomitant lower relative abundance of LNT was associated with a lower risk of sickness up to 4 months of age [8].

For respiratory pathogens, direct HMO exposure would appear less evident, and thus any putative HMO-related protection may be mediated by the intestinal microbiome [11, 40]. Yet, experimentally, direct exposure of *Streptococcus pneumoniae* to LNnT and sialyl-LNnT and subsequent infection effectively blocked its colonization in the lung of a rabbit model [41]. In a cell-based assay, LNnT and 2′FL dose-dependently reduced influenza and respiratory syncytial virus concentrations within respiratory tract cells [42].

Observational studies together with findings from preclinical models have provided first evidence for an association between HMOs and the risk of infections, mostly in a structure-function-specific way. Mechanistically, HMOs may act through multiple functions, although preclinical models highlight specific individual functions. The current studies also provide directions to be considered in future observational studies, such as timing of milk sampling and breast milk intake, etiology of infections, quantitative versus categorical HMO analysis and finally mother and infant genetics.

**HMO Composition Might Be Linked to Allergy in Infants**

Numerous environmental, including nutrition, and genetic factors affect allergies. Among them are breast milk bioactives and possibly HMOs. In a cohort of 266 Finnish mother-infant pairs with a hereditary allergy risk, 2′FL concentrations in early breast milk associated with a lower risk to manifest IgE-associated eczema at 2 years of age only in C-section born infants [43]. This observation suggests that 2′FL may influence IgE-associated eczema through the modulation of the early-life gut microbiota, known to be different in C-section-born infants compared to vaginal-born infants. A possible relation of HMOs with cow milk allergy (CMA) was studied in another cohort of 39 mothers with infants who developed CMA by 18 months of age and 41 mothers with infants without CMA [44]. An association was seen between the milk concentration of several individual HMOs [LNFP-III, 6′SL, LNFP-I, and DSLNT (DiSialyllacto-N-tetraos)] and HMO clusters with reduced risk of CMA, with LNFP-III providing the strongest association. Breast milk sampling varied over the first 6 months after
birth and this was taken into account in the statistical analysis, because HMO concentrations change dramatically during this period. Mechanistically, the authors speculate that LNFP-III might act on the immune system via dendritic cells and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin). In a preclinical food allergy model, $2'\text{FL}$ and $6'\text{SL}$ were tested and both reduced symptoms involving mast cell activity [45].

The observational studies to date have their limitations, but still provide valuable preliminary data on possible relationships between specific HMOs and risk of allergies. To appreciate such a proposed link requires replication in larger cohorts with harmonized milk sampling, stratification for mode of delivery, and evaluation of infant FUT2 and FUT3 genotypes.

**Insight from Clinical Intervention Trials with Specific HMOs**

Recent progress in industrial biotechnology has made available few individual HMOs, namely $2'\text{FL}$ and LNnT. Preclinical safety toxicity tests established their safety, and both obtained approval as novel foods in the European Union and were generally recognized as safe in the USA.

In adults, both $2'\text{FL}$ and LNnT were studied alone or in combination at different doses from 5 to 20 g/day in a placebo-controlled, blinded, randomized trial ($n = 100$). Both HMOs were well tolerated and increased bifidobacterial abundance [46].

In infants, 2 placebo-controlled, blinded, randomized, clinical intervention trials showed the growth safety and tolerance of $2'\text{FL}$ combined with either GOS or fructooligosaccharides [47, 48; Kajizer et al., unpubl.]. Infants fed with an infant formula supplemented with $2'\text{FL}$ (0.2 or 1 g/L) combined with GOS or GOS alone showed similar growth as breastfed infants up to 4 months of age ($n = 314$). In a subgroup of infants, immune markers were measured in plasma at baseline and upon stimulation of blood cells with respiratory syncytial virus. Globally the immune profile resembled that of breastfed infants when the infant formula was supplemented with $2'\text{FL}$ at the lower or higher dose [48]. Another randomized controlled infant trial showed that an infant starter formula supplemented with 2 HMO, $2'\text{FL}$, and LNnT ($n = 88$) allowed for age-appropriate growth of term born infants and was well tolerated when compared to the same infant formula without HMO ($n = 87$) [49]. Interestingly, secondary exploratory findings showed an association between feeding the 2-HMO infant formula and less-reported lower respiratory tract illnesses and medication use (especially antibiotics and antipyretics) during the first year of life and beyond the 6-month feeding period. At 3 months, the global microbiota profile shifted in the 2-HMO-formula-fed infants away from the control-formula-fed infants and towards that observed in breastfed reference infants. This shift was mainly due to increases in
*Bifidobacterium* concomitant with decreases in *Escherichia* and *Peptostreptococcaceae* [50]. A significantly higher number of infants who were fed the 2-HMO-supplemented formula showed a microbiota community structure typical for breastfed infants compared to control-formula-fed infants, who had primarily a different microbiota community structure. Interestingly, infants with a microbiota community structure typical for control-formula-fed infants had a 2 times higher risk to use antibiotics during the first year of life than those with a microbiota community typical for breastfed infants [51].

These first clinical intervention trials with specific HMOs demonstrate their growth safety and digestive tolerance. Additionally, as suggested from basic research and observational data, 2′FL and LNnT might contribute to the protection from infection-related illnesses and reduce the need for antibiotics, possibly through the modulation of the establishing early-life gut microbiota.

**Conclusion**

HMO composition is affected most notably by the maternal FUT2 and FUT3 status. This is likely due to an evolutionary selective pressure imposed by pathogens or the microbiome at large. Stage of lactation alters HMO composition possibly indicating different infant needs at different extrauterine developmental stages. However, giving birth to a preterm or term infant, who are at different developmental stages, seems not to affect the HMO composition of breast milk. Clinical observations corroborated by preclinical data and clinical intervention trials support a role for specific HMOs in immune protection, primarily from infection-related morbidity and use of antibiotics. Further clinical studies, well-designed observational studies, and especially placebo-controlled interventions are warranted to further substantiate and grow our understanding of the HMO biology and significance for infant nutrition.

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All authors are employees of Nestec Ltd.
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Abstract

Fatty acids (FAs) and fat-soluble vitamins are vital components of the human milk lipid fraction. About two-thirds of the human milk FA fraction consist of oleic, linoleic, and palmitic FAs, but the precise composition depends on maternal geography, diet, and genetics. Mothers with high fish consumption have more docosahexaenoic acid (DHA) and other ω-3 FAs in their milk, while mothers with high dairy consumption have more branched-chain FAs in their milk. Vitamins A and E are the most common fat-soluble vitamins, but milk concentrations vary, depending on maternal diet and body stores. Vitamin D is typically low or undetectable in mother’s milk and typically fails to meet the infant needs. However, trial data indicate that high maternal supplementation (6,400 IU/day) safely provides nutritionally adequate amounts of vitamin D in her milk. FA and fat-soluble vitamin levels in mother’s milk can significantly influence infant health; for example, in preterm infants, low endogenous stores of DHA paired with low levels in maternal milk may influence the risk of chronic lung disease and other inflammatory conditions. Greater attention is warranted to the variation in FA and fat-soluble vitamin content of human milk in relation to infant health.

Introduction

Fatty acids (FAs) and fat-soluble vitamins are key components of the lipid fraction of human milk. Lipid is the second-most abundant solid constituent of human milk after lactose, but it is also the most highly variable macronutrient of
Milk expressed late in a feed or pumping episode contains as much as 2–8 times more fat than at the start of the feed [1, 2]. In addition, lipid content is also reportedly lower in night and morning than afternoon or evening feeds [1, 3]. Given this high variability, accurate study of lipid-associated components requires accounting for sample lipid content.

Human milk FA composition has a core similarity (Fig. 1) but differs across populations in the abundance of many FAs, influenced by maternal diet and genetics (Table 1). High interindividual and population variability in docosahexaenoic acid (DHA) and other n-3 FAs of human milk is often observed [4–7]. Other FAs that differ between populations include the trans-FAs, and the n-6/n-3 ratio of polyunsaturated FAs (PUFAs) [6]. We have also reported that the branched-chain FAs (BCFAs) of human milk differ between populations [7]. Evidence indicates that the FA dietary profile of infants influences the risk of inflammatory conditions and neurodevelopment, and is relevant to later-life cardiovascular health [8].

The fat-soluble vitamins are also important contributors to the lipid fraction of human milk. Human milk typically has adequate levels of fat-soluble vitamins A and E to meet infant needs (Table 2), though there is variation between populations. However, human milk is typically low in vitamins D and K. Vitamin D supplementation of 400 IU/day for all infants is a current global consensus recommendation by 11 international scientific organizations for the prevention and management of nutritional rickets [9]. Fat-soluble vitamins perform important health functions and can be stored in the liver and fat tissue until required. Because they are fat soluble, these vitamins are absorbed from the diet through the small intestine along with dietary fat and are readily stored for use. Below, we briefly review the FAs, followed by the fat-soluble vitamins of human milk.

**Human Milk Fatty Acids**

*Description*

FAs are carboxylic acids with long aliphatic chains. In human milk, the FAs are found in saturated, monounsaturated, polyunsaturated, and branched forms. The preponderance of human milk FAs are long-chain FAs, which include tails of 13–21 carbons, but, human milk also includes medium-length FAs, including 8–12 carbon tails, and very-long-chain FAs, with tails of 22 or more carbons. Compared to cow’s milk, human milk contains a higher proportion of PUFAs and long-chain PUFAs (LCPUFAs) [1]. Most human milk FAs are unbranched, but human milk also contains forms of BCFAs ranging from 14 to 18 carbon
chains [7]. About two-thirds of human milk fat is composed of 3 major FAs: oleic (c18:1 n-9, a monounsaturated FA); palmitic (c16:0, a saturated FA); and linoleic (c18:2 n-6, a PUFA). While these 3 FAs are consistently dominant, the exact FA quantities and profile of human milk otherwise varies significantly between mothers and populations (Fig. 1) [6, 7].

**Fig. 1.** Abundance of fatty acids (FAs) in human milk taken from late lactation, comparing a Bolivian forager population (the Tsimane) to an urban US midwestern population (Cincinnati, OH, USA). Bar chart on the left represents the relative abundance of specific FAs in the order listed in the table to the right. In both populations, about two-thirds of the FAs consist of oleic, palmitic, and linoleic acids. In other ways, the relative abundance profile differs by population (adapted from Martin et al. [6]).

**Table 1.** Dietary factors associated with varying concentrations of fatty acids (FAs) in human milk

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Associated factor(s)</th>
<th>First author, year</th>
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</thead>
<tbody>
<tr>
<td>DHA</td>
<td>Increased with intake of fish and other DHA-rich foods; differs by population Lower in milk of mothers who deliver preterm</td>
<td>Martin [6], 2012</td>
</tr>
<tr>
<td>Branched-chain FAs</td>
<td>Dairy and beef consumption associated with higher levels of specific branched-chain FAs</td>
<td>Dingess [7], 2017</td>
</tr>
<tr>
<td>Trans-FAs</td>
<td>Higher in westernized populations</td>
<td>Martin [6], 2012</td>
</tr>
<tr>
<td>n-6:n-3 ratio</td>
<td>Higher in westernized populations</td>
<td>Martin [6], 2012</td>
</tr>
</tbody>
</table>

C/T, ratio of Cincinnati to Tsimane values. Bolded ratios indicate those that are 0.5-fold or lower (increased in Tsimane) or nearly 2-fold or higher (increased in Cincinnati).
Factors Affecting Varied Concentrations

Many FAs of human milk vary between populations, but some vary more than others. DHA is one of the most-well-studied FAs of human milk. DHA (C22:6) is a critical n-3 PUFA, and its contribution to human milk content is significantly lower in populations with low DHA dietary intake. Martin et al. [6] reported twofold greater levels of DHA and other n-3 FAs in the milk of the Tsimane, a Bolivian forager population, compared to mothers residing in Cincinnati, OH, an urban, midwestern US city. Consistent with known differences in diet, the milk of Cincinnati mothers had a significantly higher ratio of n-6/n-3 FAs, and twofold increased linoleic acid and total trans-FAs compared to Tsimane mothers. Differences in FA composition have been observed within the United States. A comparison of donor human milk from 6 milk banks across the US found a trend towards linoleic and other FA profile differences in individual milk samples donated from different regions of the United States [5]. In a study of human milk FA composition in the US over nearly 60 years, Ailhaud et al. [10] reported a threefold rise in linoleic acid between about 1945 and 2005. Thus, some of the differences now observed between populations may be due to relatively recent changes in dietary fat sources.
We compared FAs of milk from mothers in Shanghai, China; Mexico City, Mexico and Cincinnati, OH, USA and identified another intriguing population level difference: The BCFA content was highest in women residing in Cincinnati, followed by women in Mexico, and lowest among women in Shanghai. Higher dietary intake of dairy foods was significantly associated with higher levels of the BCFA iso C14:0, anteiso C15:0, and iso C16:0. Higher beef intake was associated with significantly higher levels of the BCFA iso C16:0 in human milk [7].

In addition to diet, the LCPUFA composition of human milk can also be influenced by polymorphism in maternal FA desaturase (FADS) genes, which are involved in FA elongation. In humans, FADS1 and FADS2 genes influence the ability to synthesize LCPUFAs; thereby, they modify the concentration of LCPUFA in human milk, and, specifically, the impact of fish intake on the DHA composition of human milk [11, 12]. However, FADS genes appear to be important only when the endogenous synthesis of LCPUFAs provides a compensatory advantage, that is, only when dietary intake of DHA or other LCPUFAs are limited. When the level of DHA is low in human milk, it can be dramatically increased by dietary supplementation with preformed DHA [4], regardless of the genetics of the mother.

It is noteworthy that proteomic analysis of samples from 3 global populations [13] found that FA synthesis proteins consistently increase over the course of lactation. This finding suggests that early in lactation, the FAs found in human

<table>
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<tr>
<th>Vitamin</th>
<th>Associated factor(s)</th>
<th>First author, year</th>
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<tr>
<td>Retinol</td>
<td>Lower milk concentrations in low-resource regions (Africa and Southeast Asia)</td>
<td>Samano [29], 2017</td>
</tr>
<tr>
<td></td>
<td>Mothers with low dietary intake of animal foods</td>
<td>Tanumihardjo [20], 2016</td>
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<tr>
<td></td>
<td>Premature delivery</td>
<td>Campos [23], 2007</td>
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<tr>
<td></td>
<td>Higher in early lactation</td>
<td></td>
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<tr>
<td>β-Carotene</td>
<td>Lower milk concentrations with low dietary intake of carotene-rich fruits and vegetables</td>
<td>Lipkie [26], 2015</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>Higher in early lactation, lower with premature delivery and higher parity</td>
<td>Campos [23], 2007</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Increased with dietary maternal supplementation in lactation with 6,400 IU/day</td>
<td>Wagner [18], 2006</td>
</tr>
<tr>
<td></td>
<td>Decreased with limited sunshine exposure</td>
<td>Hollis et al. [17], 2015</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>Maternal administration in pregnancy</td>
<td>Van Winckel [28], 2009</td>
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<td>Greer [30], 1997</td>
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milk may be derived more from direct blood influx (dietary sources), while late in lactation, human milk FAs may be derived more from de novo mammary synthesis (which may indicate a greater potential genetic influence).

**Physiological Effects**

Human milk FA composition may have a powerful influence on infant health. FAs regulate intracellular signaling and affect inflammatory response, cardiovascular development, and central nervous system development and function [14]. Thus, it is intriguing that infants worldwide can be exposed to significantly different FA profiles in their mother’s milk. The current Western diet may be reasonably represented by Cincinnati mothers, who have high BCFA, linoleic acid, and trans-FA contents, and low ω-3 and monounsaturated FA contents in their milk, than the milk of women in Tsimane, who represent the traditional dietary pattern. The physiological or metabolic impact these differences remains to be determined.

There is strong and growing evidence, however, that the FA content of mother’s own milk and donor milk are insufficient to meet recommendations for the health and nutrition in preterm infants. Several randomized, controlled trials have reported that supplementation of pregnant mothers with DHA contributes to longer gestation and greater infant birth weight [14]. Consistent with that finding, DHA levels are low in the milk of preterm infants [4, 15]. Further, it was found that the level of DHA measured in donor milk was also too low to support the nutritional needs of preterm infants. Preterm infants are at further risk of DHA deficiency because the accretion of DHA occurs in utero predominantly during the last trimester of pregnancy, a period that preterm infants have missed. The lack of endogenous DHA stores and low DHA levels in maternal or donor milk appear to place preterm infants at high risk of adverse outcomes during their hospitalization. In a cohort of preterm infants <30 weeks gestation, low DHA levels and increased linoleic acid/DHA ratios were associated with chronic lung disease and late onset of sepsis [15]. These and other findings provide a strong argument for attending to maternal and infant FA nutrition, including the FA composition of human milk.

**Fat-Soluble Vitamins of Human Milk**

**Description**

The fat-soluble vitamins – vitamins A, D, E, and K – are critical to infant health. Fat-soluble vitamins are absorbed from the diet through the small intestine along with dietary fat. They are readily stored for use and tend to persist in the body. Levels of fat-soluble vitamins in human milk are thus typically stable. The
quantities of vitamins A and E in human milk appear typically adequate to meet infant needs, though limited in some vitamin-A-deficient mothers (Table 2). However, vitamin D is typically absent in human milk [16], though recent data indicate that vitamin D supplementation of lactating women with 6,400 IU/day can safely produce adequate levels of milk vitamin D to satisfy the requirements of nursing infants [17, 18]. Like Vitamin D, vitamin K is also low in human milk and typically provided directly to newborns.

**Vitamin A**

Vitamin A refers to a set of related compounds that include preformed vitamin A and provitamin A carotenoids. Once consumed, these forms are converted and stored as retinol, which is used as the measure of vitamin A equivalence. Preformed vitamin A is predominantly obtained from the liver, fish oil, milk, and eggs. The provitamin A carotenoids are dietary vitamin A precursors obtained from plant foods. The most important provitamin A carotenoid is β-carotene. α-Carotene and β-cryptoxanthin also contribute some provitamin A activity.

Vitamin A plays a key role in vision, bone growth, reproduction, immunity, cell development, and skin health. Retinol and its metabolites regulate many functions in the body, including maintenance of epithelial cell integrity [19]; expression of genes that encode structural proteins, enzymes, extracellular matrix proteins, and retinol-binding proteins and receptors; and maintenance of immune function [20]. In the eye, these molecules are responsible for the differentiation of the cornea and conjunctiva, for the activity of retinal photoreceptor cells, and for changing light to neural signals for vision. Retinol and its metabolites are especially critical in early development.

Vitamin A deficiency remains a major health problem of low-resource countries and results in impaired resistance to infection, xerophthalmia, blindness, and increased risk of mortality (Table 3). As many as 190 million preschool-aged children and 19 million pregnant women suffer from vitamin A deficiency according to WHO estimates [21, 22]. Based on observations of breastfed infants in communities in which good nutrition is the norm, WHO set the recommended dietary intake for infants <6 months as 375 μg retinol equivalents (RE) per day. For an exclusively breastfed infant consuming between 650 and 750 mL per day, meeting the target intake could require a milk concentration as high as 500–600 μg/L RE/day. In healthy women with adequate vitamin A nutrition, these levels may be exceeded (Table 2) [23, 24], while in some populations, reported concentrations appear to be just meeting or modestly below the WHO-recommended intake (Table 2). In some low-resource regions, as reported in Cameroon [25], concentrations of retinol may be low among women with lim-
edited dietary sources of vitamin A. Despite finding vitamin A levels in the milk of a vitamin-A-deficient mother to be less than ideal in such populations, they are considered adequate to help reduce the risk of xerophthalmia in the infant [20]. Prenatal vitamin supplementation is effective in increasing maternal serum and breast milk concentrations [3]. In populations at high risk of vitamin A deficiency, maternal supplementation programs have focused on pregnant mothers and infants after 6 months of age. In preterm infants in high-resource countries, vitamin A supplementation of the infant during hospitalization is a priority, as preterm infants are typically born with low vitamin A stores.

The vitamin A content of human milk varies also in relation to the dietary sources of vitamin A. Analysis of provitamin A carotenoids in milk samples from China, the US, and Mexico found that the most abundant provitamin A carotenoids was β-carotene, followed by β-cryptoxanthin and α-carotene [26]. Chinese mothers had significantly higher levels of carotenoids in their milk than US and Mexican mothers, likely due to maternal dietary differences (Table 3). However, while these carotenoids contribute to the total retinol activity of human milk, they are far less abundant and efficient than retinol to support the retinol activity of human milk.

**Vitamin D**

Worldwide, studies of vitamin D in human milk have found concentrations to be below detectable levels. Inadequate vitamin D nutrition results in poor bone health and increased risk of infection. Given the absence of vitamin D in breast milk, breastfed infants are at increased risk of vitamin D deficiency, with occurrence of its most severe form – rickets – in many populations. Thus, the global public health recommendation has been to provide breastfed infants with vitamin D supplements after birth to prevent vitamin D deficiency and provide essential support for calcium absorption and bone growth [9]. Recent studies provide an alternative strategy. In a randomized, controlled trial, mothers given 6,400 IU of vitamin D during lactation achieved clinically adequate amounts of vitamin D in their milk to satisfy infant needs during early infancy (Table 3) [17]. Nevertheless, the recommended public health strategy at this time remains direct supplementation of the breastfed infant with vitamin D.

**Vitamin E**

It is comprised of 8 isoforms, including 4 tocopherol isoforms: α-, γ-, β-, and δ-tocopherol. Of these, α-tocopherol is the dominant form of vitamin E in human milk, followed by γ-tocopherol. Vitamin E is a potent antioxidant that protects against free radicals, molecules that cause cellular damage. Vitamin E is also reported to benefit immune health and serves to reduce the risk of blood clot-
α- and γ-tocopherol differ by 1 methyl group and have a similar capacity to scavenge reactive oxygen species, but γ-tocopherol may serve as a more potent antioxidant due to its capability to react with reactive nitrogen species. However, α-tocopherol is found at higher concentrations in milk and tissues than γ-tocopherol, likely due to the preferential transfer of α-tocopherol to lipid particles by liver α-tocopherol transfer protein [27].

WHO recommends an infant intake of vitamin E of 2,700 μg/day. Typically, concentrations of vitamin E in human milk from different populations meet this recommendation such that mothers are able to provide this quantity to their exclusively breastfed infants per day (Table 2). However, vitamin E levels have been reported to be considerably higher than the WHO recommendation in some populations [23, 24]. In other populations, e.g., in mothers who have delivered a preterm infant, vitamin E levels may be somewhat lower than recommended (Table 3). The possible impact of lower vitamin E levels on infant health outcomes is understudied.

**Vitamin K**

Vitamin K is responsible for the carboxylation of proteins that bind calcium, which is required for normal coagulation. Thus, vitamin K deficiency can be dangerous and result in delayed coagulation and vitamin K deficiency bleeding. For exclusively breastfed infants, the two sources of vitamin K are mother’s milk and their own endogenous gut bacteria. Human milk is a poor source of vitamin K, containing only 1–4 μg/L. The recommended dietary intake of vitamin K in infancy is 1 μg/kg body weight/day, which translates to a daily requirement of 5–10 μg/day, a requirement rarely met by human milk consumption. A single placebo-controlled trial showed that supplementing lactating mothers with high-dose vitamin K (5 mg/day) increases the level in their breast milk and is associated with an improved protein carboxylation profile (Table 3) [28–30]. Nevertheless, to assure prevention of early vitamin K deficiency bleeding of the newborn, administration of vitamin K to the newborn is the standard of care.

**Conclusion**

Exclusively breastfed infants rely on mother’s milk to meet their needs. The study of diverse populations has elucidated the compositional description of human milk components and basic understanding of factors that influence human milk composition. In relation to the FAs, 3 FAs consistently form the major part of the FA fraction (oleic, palmitic, and linoleic acids). Nevertheless, considerable variation is seen between populations in the quantity of specific FAs. This varia-
tion is largely due to maternal dietary differences, though genetic polymorphisms can contribute when dietary intakes of specific LCPUFAs are limited. In relation to the fat-soluble vitamins, vitamins A and E are typically present in robust quantities in human milk, though some high-risk mothers (e.g., those who deliver preterm or live in resource-poor countries) have lower than recommended levels. However, vitamins D and K are typically low in human milk, and infant supplementation is the recommended strategy for assuring adequate infant nutrient status. Our review of the literature suggests that the most critical scientific questions are: Do variable levels of FAs in human milk impact the health or development of infants? This question is also pertinent for some of the fat-soluble vitamins of human milk. Greater attention is also warranted to maternal supplementation as an approach to modifying FA levels and fat-soluble vitamins in human milk, particularly in relation to DHA and vitamin D.

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Abstract

Most B vitamins and vitamin C are among the nutrients in milk most strongly affected by maternal status and/or dietary intake. Recent analytical methods are more efficient and valid, revealing major differences in water-soluble vitamins across population groups. An inadequate supply in milk can be detrimental to the breastfed infant’s health and development although cutoff points below which risk is increased are often uncertain, and little attention has been paid to adverse effects of low milk water-soluble vitamins on infant health and function. Concentrations change during lactation: thiamine, niacin, and pantothenic acid increase; B₆, B₁₂, and ascorbic acid gradually decrease; while riboflavin concentrations are stable, as is choline after an initial increase. Folate fluctuates until stabilizing in late lactation. Water-soluble vitamin concentrations in milk are also influenced by maternal supplementation, and, for some, by parity, preterm delivery, smoking, and maternal illness. However, there is relatively little change in concentrations during a feed nor is diurnal variation a major influence. Reported concentrations are used to set adequate intakes for infants and incremental requirements for lactation. However, the status of available data is poor due to the small number of participants in most studies, uncertainties about maternal nutritional status, and variable times of milk collection postpartum.

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Introduction

All of the water-soluble vitamins are essential for the health, development, and survival of the breastfed infant. During the first 6 months of life, it is recommended that human milk is the sole source of nutrients for infants, and that it should remain an important source through at least 2 years of age. However, relatively little attention has been paid to the micronutrient content (quality) of milk from women consuming poor diets. Due in part to improvements in analytical methods, there is increasing evidence that the water-soluble vitamins are among the nutrients most likely to be secreted in reduced amounts if the mother’s status and/or intake are low.

In this article, for each of the water-soluble vitamins we summarize available information on the forms and concentrations in milk; the effects of poor maternal status on levels in milk and subsequent effects on infant status and function; normal changes during lactation; the effects of maternal interventions to increase concentrations in milk; and of other factors such as parity and smoking. We present comparative values for concentrations in milk from lower income countries measured by an efficient ultra-or high-performance liquid chromatography tandem mass method developed in our laboratory [1] and samples collected by collaborators from unsupplemented women at similar stages of lactation.

Much of the information presented is discussed in more detail in our recent series of 7 articles on micronutrients in human milk [2]. There, we discuss the paucity of data on milk micronutrients and the lack of reliable values for setting the adequate intakes (AIs) by the Institute of Medicine (IOM) and other countries.

Forms in Human Milk, Effects of Maternal Status, and Relationship to Infant Function

Thiamine
Thiamine (B₁) and its phosphate esters are crucial for normal carbohydrate, nucleic acid, and amino acid metabolism, for example, decarboxylation of α-keto acids, the transketolase reaction in the pentose phosphate pathway, synthesis of the neurotransmitter acetylcholine, and nerve impulse transmission. The predominant forms in human milk are thiamine monophosphate (~60%) and free thiamine (~30%), plus a small amount of thiamine triphosphate. The global prevalence of thiamine deficiency is uncertain but is likely high in populations where grains are polished and animal source food intake is low. For ex-
ample, in a nationally representative sample from the 2014 Cambodian National Micronutrient Survey, at 6–12 months postpartum, 27% of mothers and 38% of infants were thiamine deficient [3]. Maternal deficiency is rapidly reflected in low milk thiamine, with concentrations <0.12 mg/L associated with poor infant growth in an older Malaysian study. Based on analyses in our laboratory, we find that in Cambodia, Ghana, India, the Gambia, Indonesia, and Peru, milk concentrations are only 60–70% of the 0.21 mg/L IOM-accepted value, which was based on a 1985 report. Beriberi presents in breastfeeding infants who are thiamine deficient due to severe maternal deficiency, but it is much less common than 50 years ago. However, even in infants with marginal status, mortality peaks at age 3 months [3], and growth and auditory, motor, and language development was retarded years later in Israeli children who consumed formula lacking the vitamin when they were infants [4]. A 2004 study reported that thiamine deficiency was common among breastfeeding sick infants in Laos and associated with a higher risk of mortality [5].

Riboflavin

As part of the coenzymes flavin adenine mononucleotide and flavin adenine dinucleotide (FAD), riboflavin (B2) is required for energy production, fatty acid and amino acid synthesis, DNA repair, folic acid activation, conversion of hepatic tryptophan into niacin, and production of glutathione, a free radical scavenger. Humans need a constant dietary supply of vitamin B2 (from meat, milk, and dairy intake) as it is inefficiently stored. The predominant forms in human milk are FAD (60%), free riboflavin (30%) and other flavin derivatives [6]. A concentration of 0.35 mg/L was accepted by the IOM for setting the infant AI although this was based on samples from only 5 mothers. Milk riboflavin concentrations are highly dependent on maternal intake of the vitamin [7]. Maternal intake and status are strongly dependent on usual intake of dairy products, although studies of the global prevalence of inadequacy are relatively few. About 75% of young women and 95% of adolescent girls had poor riboflavin status in the UK diet survey (mostly low dairy product consumers), and the prevalence of deficiency was reported to be high in Canada, Europe, and in an Irish National Survey [8]. In pregnant and lactating women in Guatemala, Nepal, and India, riboflavin deficiency occurred in 77–85% although these were older data and not nationally representative. Our analyses show concentrations in milk are only 10–20% of the values used to set the AI in samples from Bangladesh, Kenya, Peru, Cambodia, Indonesia, and the Philippines. Poor riboflavin status has been reported in an older study of Gambian infants whose mother’s milk was low in the vitamin [9], but the effects of deficiency on infant development and status are not known.
Niacin
Niacin (B3) is the collective term for the interconvertible nicotinic acid and nicotinamide, the building blocks for nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes are involved in oxidation-reduction reactions (e.g., electron carriers for intracellular respiration), catalyze the oxidation of fuel molecules as codehydrogenases (NAD), or serve as a hydrogen donor in the reductive processes of fatty acid synthesis (NADP). Humans are capable of hepatic in vivo nicotinic acid production from tryptophan where 60 mg produces 1 mg niacin. This ratio is not certain for the infant. Forms of niacin in human milk include nicotinamide, NAD, NADP, nicotinamide riboside, and nicotinamide mononucleotide. The global prevalence of niacin deficiency is not known although it is likely to be greater where maize is not lime treated, or the staple diet is starchy, low-protein foods. The IOM accepts 1.8 mg/L as the normal milk value, but this is based on one old UK study including 23 women from 16 to 244 days lactation, where the range was 1.2–2.8 mg/L. We find that values are <20% of this concentration in California, Cambodia, Indonesia, Bangladesh, the Philippines, India, Peru, Malawi, and Kenya. Surprisingly, concentrations were highest in Ghana and the Gambia, where they approached 60% of the AI value.

Pyridoxine
Vitamin B6 (pyridoxal, pyridoxine, and pyridoxamine) is a cofactor for >100 enzymes required for amino acid metabolism, glucose synthesis, glycogen breakdown by phosphorolysis, and steroid (and other nuclear-acting) hormone regulation. The form in milk is mainly pyridoxal (75%) with small amounts of pyridoxal phosphate, pyridoxamine, and pyridoxine. Concentrations of B6 vitamers in milk are positively correlated with maternal status based on the assessment of plasma pyridoxal 5′-phosphate (or PLP) [10]. The global prevalence of deficiency in women is unknown. It has been reported as high in US and Canadian surveys although there is uncertainty about the cutoff point that should be used. Older studies reported that 40% of Egyptian mothers had milk B6 <0.1 mg/L associated with lower birth weight, abnormal infant behavior, and less responsibility in the mothers [11]. About 15% of exclusively breastfed Finnish infants had poor B6 status at age 6 months associated with slower growth. In the USA, although mothers consumed the recommended dietary allowance (RDA) for B6, those with higher intakes had more in their milk and their infants had higher scores on the Brazelton Neonatal Behavioral Assessment Scale [12]. The IOM reported a value of 0.13 mg/L from 19 participants at 3 weeks to 30 months postpartum. We find concentrations <60% of this value in samples from Cambodia, Bangladesh, Peru, Indonesia, the Philippines, Ghana, and the Gambia. Interest-
ingly concentrations in a Californian group of lactating women were 230% of the AI, possibly because vitamin B₆ is included in relatively high amounts in many prenatal supplements.

**Cobalamin (B₁₂)**

B₁₂ in biological materials is predominantly present as coenzyme B₁₂, in which the axial group is either occupied by a methyl group or an adenine nucleoside connected by a cobalt-carbon. These forms are important enzymes in the folate-dependent methylation of homocysteine to methionine and the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A (1-carbon metabolism), and DNA synthesis. The vitamin is tightly bound to the binding protein apo-haptocorrin in milk, which can interfere with cobalamin measurement; this problem has been avoided in recent assays [13]. In human milk, vitamin B₁₂ is mainly present as methylcobalamin and 5′-deoxyadenosylcobalamin with small contributions of hydroxo- and cyanocobalamin. The IOM accepted a concentration of 0.42 µg/L based on a small sample of Brazilian women and possibly invalid analytical methods, but concentrations in a small group of Californian and Danish samples were close to the AI in the more recent report. Analyses in our laboratory reveal milk B₁₂ concentrations ranging from about 20% (Kenya, Guatemala) to 50% of the AI in most low-income countries, but AI values were higher in Malawi (60%), Bangladesh (80%), and Ghana (120%), possibly due to the consumption of small dried fish.

It has long been established that maternal vitamin B₁₂ deficiency caused by strict vegetarianism or undiagnosed pernicious anemia produces symptoms of deficiency in exclusively breastfed infants around age 3–4 months. These include growth stunting and severely reduced head circumference, apathy, inability to consume complementary foods, and motor and cognitive delays which do not completely resolve in about 50% of cases after intramuscular or high-dose treatment [14]. Milk B₁₂ is lower even in marginally depleted mothers, and there is a high prevalence of marginal B₁₂ deficiency in the many populations that consume low amounts of animal source foods. A positive correlation between maternal intake of B₁₂ and milk B₁₂ has been demonstrated in several countries including Kenya, Guatemala, and Mexico. B₁₂ behaves somewhat differently from other B vitamins in that infant status may depend more on the accumulation of liver B₁₂ stores in utero than on maternal intake during pregnancy or lactation. For example, even when B₁₂-depleted Bangladeshi mothers were given a relatively high dose (250 µg/day) from 18 weeks of pregnancy through 3 months of lactation, infant status at 3 months was still correlated with maternal serum B₁₂ in early pregnancy [15]. This suggests a long-term ef-
fect of maternal status on infant status probably via in utero accumulation of the vitamin in fetal liver. The remaining challenge is to document if there is a causal link between marginal maternal $B_{12}$ status, milk $B_{12}$, and impaired infant development.

**Folate**

Folate in its coenzymatic form is needed for the transfer of 1-carbon atom groups such as formyl (CHO), methyl (CH$_3$) or formimino-functions (CH=NH) in amino acid metabolism and in purine and pyrimidine synthesis in nucleic acid formation (DNA and RNA). The main form in milk is N-5-methyl tetrahydrofolate. The IOM accepts 85 μg/L as the average milk folate concentration. Folate behaves differently from the other B vitamins in that the mammary gland maintains the concentrations in milk at the expense of maternal stores [16], thus neither maternal intake nor status is related to milk folate concentration.

**Biotin**

Biotin is a component of carboxylase enzymes that are vital for amino acid metabolism, gluconeogenesis, fatty acid biosynthesis, and odd-chain fatty acid catabolism. Forms in milk include biotin and its metabolites bisnorbiotin and biotin sulfoxide. In the genetic condition BIOT, lack of biotinidase leads to biotin deficiency resulting in symptoms such as seizures, hypotonia, and respiratory problems within a few months of birth. However, biotin is ubiquitous in the diet, and there are no reports of low biotin in milk due to maternal deficiency.

**Choline**

Choline is a precursor for the neurotransmitter acetylcholine and for betaine. As a coenzyme, choline is vital for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism. Human requirements for choline are relatively large, and the IOM accepts an estimate of 160 mg/L milk from the two available studies. The main forms in milk are water-soluble free choline, phosphocholine, and glycerophosphocholine, with little contribution (~10%) of fat-soluble phosphatidylcholine (lecithin) and sphingomyelin. Maternal serum choline is correlated with milk and infant concentrations, and milk choline is also affected by MTHFR genotype [17]. It is likely that adequate milk choline is vital for the normal development of the infant based on the fact that large amounts of its oxidation product, betaine, are excreted during the first year, and a lower choline status of young children has been associated with stunting [18].
Ascorbic Acid (Vitamin C)

This water-soluble, antioxidant electron donor supports the immune system, stimulating leukocytes, increasing antibody production, and stimulating production of interferons. It is required for hydroxylation of amino acids in collagen. In milk, the main forms are ascorbic acid and dehydroascorbic acid, totaling an estimated 1.8 mg/L in the three studies used by the IOM to set the AI. Low intakes of this vitamin are common where fruit and vegetable availability fluctuate by season, so milk concentrations may be greatly seasonally affected [19]. Consuming a rice and lentil complementary food with human milk to increase ascorbic acid intake did not improve the inhibitory effect of phytate on iron absorption by Bangladeshi infants and young children. Adverse effects of low milk ascorbic acid on the infant have not been reported.

Concentration Changes during Lactation

Concentrations of all water-soluble vitamins occur during the postpartum period, as summarized elsewhere by Dror and Allen [20]. Thiamine, niacin, and pantothenic acid concentrations in milk increase throughout the first few months of lactation. Riboflavin concentrations are fairly constant in well-nourished mothers within at least the first 3 months of lactation. Indian women of low socioeconomic status showed increasing riboflavin concentrations to a peak at 2–4 months and a decrease by 5–6 months [21]. Similarly, vitamin B₆ concentrations increase 300–400% during the first weeks postpartum and fall later in lactation, raising concern that human milk alone may be insufficient to meet the infant’s requirements at 6 months of age [22].

A systematic review of studies using valid methods of B₁₂ analysis indicate that B₁₂ concentrations are very high in the colostrum and then fall in the first few weeks postpartum and are stable until about 2–4 months [23]. In well-nourished Danish mothers, B₁₂ concentrations were higher at 9 months than they were at 4 months, primarily due to an increase in haptocorrin [24]. Also, in well-nourished Danish and Norwegian women, infant plasma B₁₂ decreased and methylmalonic acid increased inversely with milk B₁₂ concentration, such that the ability to supply sufficient B₁₂ to infants in mid-lactation has been questioned, even for well-nourished mothers [25]. Formula-fed infants usually have much higher serum B₁₂ concentrations than those exclusively breastfed.

Unlike the situation for most B vitamins, folate is low in colostrum, increases during the next few weeks, peaks at 2–3 months, falls between 3–6 months, and then is stable until late lactation [26]. Total choline also increases between 7 and
22 days after delivery and then remains unchanged in mature milk; however, free choline falls between 12 and 180 days [27]. Concentrations of vitamin C are highest in colostrum and fall during lactation [26].

**Effects of Maternal Supplementation or Food Fortification**

The B vitamins differ in the extent and rate at which they increase in milk in response to maternal supplementation. The effects of acute supplementation were studied in Bangladeshi women who received a single dose of ~1× the RDA of multiple micronutrients followed next day with a single dose of 2× RDA before breakfast. Concentrations of vitamins were measured in milk at each feed during the next 24 h [28]. Acute responses were observed for thiamine, riboflavin, and pyridoxal. Although only about 1% of the supplemental vitamins were secreted into the milk, this amount was equivalent to 2–99% of the AI for infants aged 0–6 months. Maternal supplementation with micronutrients for 6 months likewise showed that only a few percent of the supplemented water-soluble vitamins appeared in the milk [29] (Fig. 1). In this study, conducted in Malawi, HIV-positive mothers were enrolled to evaluate the impact of antiretroviral therapy and micronutrient supplements on maternal morbidity and HIV transmission in breast milk. The lipid-based micronutrient supplement provided daily within a few days of delivery increased milk thiamine, riboflavin, nicotinamide, B₆, and B₁₂ within 2 weeks (Fig. 1). Interestingly, concentration had not increased further at 24 weeks. ARV treatment eliminated the positive effects of lipid-based micronutrient supplements on milk B vitamins.

Studies have also evaluated the impact of single B vitamins on the amounts secreted in milk. In the case of thiamine, a rapid response was reported in population groups with a high prevalence of deficiency. In a small trial in thiamine-deficient Cambodian women, a dose of 100 mg for 5 days increased milk thiamine from 180 (85–359) to 503 (360–808) nmol/L on day 6 [30]. An intervention that supplied Cambodian mothers with poor status a thiamine-fortified fish sauce for 6 months, starting in pregnancy, increased thiamine in milk from 144 to 207 μg/L 4 months postpartum and also improved infant thiamine status [31]. In general, maternal supplementation has a positive effect on milk riboflavin concentration [32]. Maternal supplementation of deficient mothers in the Gambia improved their status, milk riboflavin, and infant status [9]. B₆ supplementation also increases milk concentrations within hours [33], and there was a dose response when mothers were supplemented with pyridoxine hydrochloride from 0 to 20 mg for 3 days [34]. Phosphatidyl choline supplements increase milk free choline and phosphocholine [17]. In Canada, maternal folic acid supple-
mentation, especially if >400 µg/day, increased folate in milk by 18% and free folic acid by 126%, and reduced 5-methyl tetrahydrofolate by 19% [35].

More maternal supplementation studies have provided B₁₂ in lactation compared to other B vitamins, due to the well-documented severe and potential irreversible symptoms of deficiency that occur in the exclusively breastfed infants of deficient mothers. A recent systematic review showed that supplementation of mothers during pregnancy and early lactation, or during months 0–6 of lactation, increased milk B₁₂ significantly compared to placebo or non-placebo con-

**Fig. 1.** Mean (SEM) concentrations of water-soluble vitamins in human milk 2, 6, and 24 weeks after maternal multiple micronutrient supplementation and/or ARV treatment. The 100% reference line is the value in nonsupplemented women at each time point. *p < 0.05, **p < 0.001, control vs. supplemented groups. Women received daily lipid-based nutrient (LNS) supplementation and/or antiretroviral (ARV) treatment for 24 weeks postpartum. AI, adequate intakes. Modified from Allen et al. [29].
Doses were 50 µg/day in pregnancy and the first 3 months of lactation, or 3–1,000 µg/day during lactation. There was little difference in the level of milk response across this range of doses, probably due to the well-known inverse relationship between the efficiency of active absorption and B\textsubscript{12} dose. Among all the intervention studies conducted to date, the most remarkable increase in milk B\textsubscript{12} occurred in Cameroon within 1 year of a wheat flour fortification program [36], possibly reflecting more efficient absorption of consistent low doses of the vitamin.

Other Factors

Other factors that can affect water-soluble vitamins in milk include parity, preterm delivery, diurnal variation, smoking, and illness, although these effects have not been studied systematically, and available information is patchy, as summarized elsewhere [20]. There is virtually no information on how any of these factors affect levels of thiamine. Parity does not affect milk riboflavin or folate. Both higher and lower B\textsubscript{6} concentrations have been reported in preterm milk. In the two studies examining whether preterm delivery affects B\textsubscript{12} in colostrum, transitional milk, or preterm milk, there was no relationship. Choline is lower in preterm milk whereas vitamin C is more concentrated. Choline in milk is related to maternal inflammation, prolactin (positively), and cortisol (negatively). Smoking reduces milk vitamin C.

While significant yet small differences were found for concentrations of thiamine, riboflavin, and nicotinamide based on timing of collection during a feeding, pyridoxal and B\textsubscript{12} were unaffected. Diurnal variability is small compared to interindividual variation in concentrations. The best time of collection for reflecting average daily concentrations was observed during the afternoon, but maternal supplementation can affect this natural fluctuation [28]. Milk folate is higher in the afternoon and evening than in the morning.

Conclusions

Water-soluble vitamins in human milk have been measured in only a small number of studies, and the validity of the values used to set the intake recommendations for infants and lactating women is uncertain. However, it is clear that the milk concentrations of these vitamins in all the low- and middle-income countries we have measured are substantially lower than the values used to set the AIs – in some countries substantially so. The global prevalence of poor water-soluble vitamin status is almost unknown, as are the effects of low milk concentrations on infant growth, function, and development. We also need to learn
the most effective ways of improving maternal, milk, and infant status including the timing of supplements and the effectiveness of food fortification. Currently, we are conducting a 4-country study to establish reference values for micronutrients in milk from well-nourished women across lactation and their relationship to maternal diet and maternal and infant status indicators.

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References


Abstract

Human milk contains a wide variety of bioactive components, including long-chain fatty acids, complex oligosaccharides, and bioactive proteins. More recently, it was discovered that breast milk also contains exosomes, i.e., microvesicles containing microRNAs (miRNAs) with sizes of ~22 nucleotides. Several of these miRNAs have been shown to originate from the mammary gland, and many of them are involved in cellular development and immune function. Exosome-mediated transfer of miRNAs is a novel mechanism of genetic exchange between cells. It is therefore possible that exosomes in milk may survive digestion and deliver miRNAs to intestinal cells, or, if transferred into the bloodstream, to cells in other tissues. In vitro work has shown that exosomes and their miRNA cargo can survive proteolytic digestion and that intestinal epithelial cells take up the exosomes and deliver them to the nucleus. Research on human adults consuming cow milk has shown that major bovine milk miRNAs are found in the circulation postprandially, further suggesting that exosomes can resist conditions in the gastrointestinal tract and be delivered to the systemic circulation. Thus, it is possible that milk miRNAs may transfer genetic material to the infant and thereby affect gene transcription and regulation of cellular events in several tissues.

Introduction

Human milk provides many benefits to the breastfed infant resulting in significantly better short- and long-term outcomes as compared to formula-fed infants. These benefits are likely achieved by a well-balanced supply of nutrients and a
wide variety of bioactive components in breast milk. These components include long-chain fatty acids (e.g., DHA), complex oligosaccharides, bioactive proteins (e.g., immunoglobulins, lactoferrin, and osteopontin), nucleotides, and lutein. By various mechanisms that have been extensively studied, they protect the infant against infections and stimulate brain development and visual function.

A novel and very fundamental mechanism to potentially affect a multitude of cells and functions in the breastfed infant is the transfer of microRNAs (miRNAs) via human milk. Such miRNAs may affect gene transcription in the small intestine and possibly other organs and therefore have the capacity to regulate many physiological functions (Fig. 1). The miRNAs are packaged in particles, exosomes, which were recently found and characterized in breast milk [1].

**MicroRNAs**

The finding of RNAs of very small size was initially believed to be “metabolic by-products,” but it was soon found that this group of miRNAs, which only contain 4–20 nucleotides, exerts important regulatory roles in a large variety of cells. They are formed very specifically and their biogenesis involves 3 steps: (1) miRNA genes are transcribed by RNA polymerase II or III into primary miRNA transcripts with (2) subsequent cleavage by the microprocessor complex Drosha-DGCR8 in the nucleus, resulting in a precursor hairpin (pre-miRNA), which is exported out of the nucleus for (3) final processing into mature miRNAs by the enzyme RNase Dicer [2]. miRNAs bind through partial sequence homology to the 3′-untranslated region of target mRNAs and cause either translational block or mRNA degradation. While most of the early research on miRNAs focused on their significance in various forms of cancer and biomarker discovery, it was subsequently found that they are also present in various foods, such as milk, meat, and plants.

**Exosomes in Milk**

Exosomes are small extracellular vesicles about 30–100 nm in size and are produced by a variety of cells including macrophages, lymphocytes, dendritic cells, and epithelial and tumor cells [3]. They are found in physiological fluids such as saliva, plasma, and urine [4]. It is well known that exosomes are important in cell-cell signaling, but their physiological significance in vivo is less known. Early studies suggested promising roles in immunotherapy and cancer therapy, but this is a rapidly advancing field and many clinical trials are ongoing.
The presence of exosomes in breast milk was first described by Admyre et al. [1], who also showed that isolated milk exosomes could affect immune responses of peripheral blood mononuclear cells (PBMCs) and T-regulatory cells. Exosomes contain specific marker proteins, such as MHC classes I and II, CD63, CD81, and CD86 [1], which may be involved in cell recognition, but, more importantly, miRNAs have the capacity to regulate transcriptional activity. It was subsequently shown that breast milk exosomes contain RNA, and that this RNA is short (20–35 nucleotides) and does not contain ribosomal RNA [5]. Kosaka et al. [6] studied miRNA expression in breast milk by microarray analysis and found large numbers of miRNAs (281 of 723 human miRNAs known at that time) and in particular high levels of immune-related miRNAs during the first 6 months of lactation. Among the major miRNAs detected were miR-181a and miR-181b, which are regulators of B-cell differentiation and CD4+ T-cell selection; miR-155, a regulator of T- and B-cell maturation and immune responses; the miR-17–92 cluster, which is a ubiquitous regulator of B-cell, T-cell, and monocyte development, and miR-125b, a negative regulator of TNF-α production, activation, and sensitivity. They found that miRNA expression in breast milk samples from the same mother did not vary greatly with time after birth. Treatment of breast milk with RNAse had little or no effect on the miRNAs, nor...
had freeze-thaw cycles or exposure to acidic pH (pH 1), suggesting that they may survive in the gastrointestinal tract. It was proposed that their presence in exosomes may shield them from the effects of low pH and digestive enzymes.

Further deep sequencing of breast milk exosomes revealed 602 unique miRNAs, that they are in a narrow size range (20–24 nucleotides), and that many of them are involved in immune function [7]. Although they only analyzed milk from 4 mothers, their results suggested low intersubject variability. They further exposed raw milk with added synthetic exogenous (not milk-related) miRNAs to various harsh conditions (prolonged room temperature, freeze-thawing cycles, RNase incubation, and high temperature exposure) and found that the exogenous miRNAs were rapidly degraded, but not the endogenous milk miRNAs. This strengthens the hypothesis that their presence in exosomes in milk protects them from digestion.

**Origin of Milk MicroRNAs and Variation during Lactation**

To explore the origin of human milk miRNAs, Alsaweed et al. [8] used next-generation sequencing to analyze miRNAs in human milk cells and fat, and compared them to miRNAs in maternal PBMCs and plasma. They found a strong association in miRNA profiles between human milk cells and fat, but miRNAs in PBMCs and plasma were distinctly different. Since most cells in mature breast milk are epithelial cells, they suggested that the milk miRNAs primarily originate from the mammary epithelium with a smaller contribution from the maternal circulation [8]. They also analyzed infant formulas and found them to contain very few human miRNAs and at very low abundance.

The variation in miRNA content and composition during the lactation period has been characterized [9]. Total miRNA concentration was similar at 3 lactation stages (2, 4, and 6 months) as were the top 20 known miRNAs and the number and expression of known miRNAs in human milk fractions. However, about one-third of the known miRNAs were differentially expressed during the first 6 months of lactation, with more pronounced upregulation at 4 months. The authors concluded that although the total miRNA quantity delivered to infants does not change during the first 6 months of lactation, the composition is altered, particularly at 4 months, which may reflect remodeling of the mammary gland in response to partial weaning [9]. We also compared total miRNAs during lactation and found little variation, but we did find that several of the top 15 milk miRNAs showed considerable variation [10]. This may be due to the limited number of subjects/samples analyzed in these studies. Interestingly, 11 out of the 15 top miRNAs found in our study of US women were also found in the
study of Australian women [9]. Similarly, all top 10 miRNAs identified by Zhou et al. [7] in milk from Chinese women were found among the top 30 miRNAs analyzed in our study, indicating that the major miRNA species in human milk are not much affected by maternal ethnicity.

The composition of preterm human milk is known to be different than that of term milk, and in a recent study metabolism-related miRNAs were found to be affected by premature delivery [11]. There were 113 miRNAs with significant expression differences between preterm and term milk lipids, most of which have been described in earlier studies. The authors selected the 15 miRNAs with the most significant differences for functional analysis and found that the most prominent mRNA targets are involved in cellular nitrogen metabolism, biosynthesis, catabolism, symbiosis, and viral processing. The pathway with the most significant enrichment in miRNA targets in preterm milk was glycosphingolipid biosynthesis [11], which is interesting as glycosphingolipids are involved in neurodevelopment [12]. It was also found that miRNAs in term colostrum were similar to those in term mature milk, and that both differed substantially from preterm milk. The authors suggested that premature delivery in itself may affect the miRNA composition in milk as 21 of 26 miRNAs were significantly related to premature delivery, and 6 of them correlated with the delivery method (cesarean section or vaginal delivery). The possibility of miRNA production in the cell nucleus being affected by the rapid shift in maternal hormones during the late preterm and early postpartum period was also discussed.

**Effect of Digestion on Milk Exosomes**

Whereas some previous in vitro studies indicated that milk exosomes and their miRNA cargo can resist harsh conditions like acid, boiling, and RNase treatment, these conditions are not the same as those in the recipient infant being breastfed. We have exposed breast milk to a gastric pH commensurate with an infant stomach pH (4.0) and incubated with pepsin for 20 min, then adjusted the pH to 7.0, similar to what would happen with the secretion of pancreatic fluid, followed by incubation for a further 30 min with pancreatin [10]. We found that normalized reads of the top 15 miRNAs isolated from human milk exosomes before and after in vitro digestion were very similar, and that they had similar overall abundance distribution.

Bovine milk was also found to contain microvesicles carrying miRNAs [13], among them miR-101, miR-125b, miR-150, miR-223, miR-24-1, and miR-93. To explore whether humans can absorb biologically meaningful amounts of miRNAs from commercial cow milk, Baier et al. [14] gave varying volumes of
milk to 5 adult volunteers and analyzed 2 major milk miRNAs (miR-29b and miR-200c) in PBMCs. Plasma time curves showed an increase in miR-29b, which was highest at 4 h and had returned to baseline by 9 h. The AUC (area under the curve) showed a linear dose-response to the volume of milk ingested, suggesting that milk-based miRNAs indeed can survive digestion and be absorbed and thus transferred to various cells and tissues. Studies in human Caco-2 cells and rat IEC-6 cells suggest that the intestinal uptake of exosomes is mediated by endocytosis [15].

The bioavailability of dietary miRNAs is controversial. The study by Baier et al. [14] strongly suggests that part of milk-borne miRNAs is absorbed, which may be explained by their presence in milk exosomes. As described above, the structure of the milk exosome may at least in part protect the miRNAs from the conditions in the gut. When 2 miRNAs from broccoli were analyzed in plasma from subjects participating in a broccoli feeding study, they were below the detection limit [14]. It is thus possible that the miRNAs in plant-based diets are more vulnerable to digestion, or that the human intestine is less capable of absorbing plant exosomes. Experiments in various knockout and knock-in mouse models addressing this research question are less convincing [16]. When miR-30b was overexpressed 134 times in transgenic mice, the authors found no effect of the increased level of this miRNA in milk on pup tissue levels [17]. As pointed out by Melnik et al. [16], they did not assess whether the additional miRNA in the milk was present in exosomes, which affects its stability. Since the extra miR-30b was substantially lower in stomach contents of the mice than in the milk, it is possible that the overexpressed miRNA was in another milk compartment and more vulnerable to proteolysis and possibly also less bioavailable. Knockout mouse pups lacking miR-375 and miR-200c/141 and nursing wild-type mothers were used by Title et al. [18] to assess transfer of these miRNAs to the systemic circulation. They found a very small increase in plasma levels of these miRNAs and concluded that milk miRNAs do not play a genetic regulatory role in newborn mammals. However, these miRNAs are involved in control of endocytotic events and epithelial cell function, and hence mechanisms for exosome/miRNA uptake in the pups may have been impaired [16], making this a less convincing model.

Cellular Uptake of Exosomes/MicroRNAs

Studies have shown intestinal uptake of undigested milk exosomes in pigs and rats [19, 20]. We exposed human intestinal epithelial crypt-like cells to exosomes isolated from breast milk that was undigested or subjected to in vitro digestion as described above [10]. The digestive fate is important for major nutrients like
proteins and lipids, but also for extracellular vesicles, such as human milk exosomes. Demonstration of intestinal cell uptake after gastric/pancreatic digestion would provide support for the notion that exosomes are vehicles for transfer of genetic material from the mother’s milk to her offspring. We found that the protein profiles of the exosomes exposed to digestion at both pH 4 and pH 2 were similar to those of undigested exosomes. Since we have previously shown that purified lactoferrin from human milk is degraded at pH 2 [21] and lactoferrin as a component of the exosome protein is not, it is possible that the partial compartmentation of lactoferrin into exosomes protects it from proteolysis and facilitates its uptake into the enterocyte. Using fluorescent dyes and confocal microscopy, we were able to show that the cells could readily take up exosomes from both untreated and in vitro digested breast milk at 0.5 and 2 h. At both time points, about 10% of the internalized exosomes localized to the nucleus, suggesting novel mechanisms for nuclear gene regulation conferred by human milk exosomes. Although speculative, the advantages of the delivery of gene expression regulators via breastfeeding is apparent: (1) exosomes provide a boundary that protects molecules from being attacked by low pH and a high enzymatic activity environment; (2) efficient batch recognition and internalization by the intestinal epithelium; and (3) bolus delivery for higher local concentration and therefore efficacy of action.

**Functions of Breast Milk MicroRNAs**

We ranked the relative abundance of all detected miRNAs in our study [10] and found 630 miRNAs of which 288 were more abundant. We noted that miR-22-3p is consistently (from early to late lactation, undigested vs. in vitro digested) detected as the most abundant miRNA species in our study, accounting for ~20% of all top 296 miRNA read counts. miR-22-3p is an immune-related miRNA abundant in liver, targeting transcription factor 7, an important effector molecule in the Wnt pathway, which increases expression of enzymes in the liver gluconeogenic pathway, and therefore is a therapeutic target to treat insulin resistance and type 2 diabetes [22]. Its identification in human milk exosomes expands its established role of modulating carbohydrate metabolism to a potential new domain of benefits in postnatal developmental programming.

We used TargetScan to obtain target genes for the 5 most abundant miRNAs in our expression profile data. hsa-miR-30d-5p has a small number of 21 targets, whereas the other 4, hsa-miR-22-3p, hsa-miR-148a-3p, hsa-141-3p, and hsa-miR-181a-5p, all have more than 600 predicted transcripts with conserved target sites. There are 406 genes that are targets for more than 1 of these latter
4 miRNAs, and transcription regulation-related molecular functions are the most highly enriched, showing that miRNAs transferred from the mother can influence production of functional proteins that regulate the infants’ DNA decoding to RNA, which is also reflected by the highly enriched transcription-related gene ontology biological process terms. Synapse localization is also strongly enriched among these genes. Synapse formation, stabilization, and plasticity are key features of neuronal development [23], and many miRNAs have been shown to be involved in synapse plasticity [24]. We therefore surveyed miRNAs that have positive impact on mammalian synapse development, and half are present in the top 288 group of human milk exosome miRNAs. The presence of these miRNAs suggests that brain development in early life may benefit from these functions.

We then surveyed the miRNAs identified in our study for the previously reported 59 immune-related pre-miRNAs [7] and identified 57 of them, including 50 in the top 288 miRNA group [10]. We also curated a list of 86 immune-related miRNAs based on the literature, and this covered 29 of the 59 miRNAs previously identified [7]. Among the 86 immune-related miRNAs, 65 (75.6%) are present in the top 288 group reported in our study. Discovering a large number of these additional immune-related miRNAs in human milk exosomes, including miR-29a-3p, miR-29b-3p, miR-22-3p, miR-181c-5p, miR-181a-5p, miR-181a-3p, miR-16-5p, miR-26a-5p, and miR-34a-5p, reinforces that human milk exosomes are a rich source of immune-related miRNAs.

Involvement of milk miRNAs in brain development was also suggested by Carney et al. [11] who found that the pathways with the most significant enrichment in miRNA targets from preterm milk are biosynthesis of glycosphingolipids and cell membrane function. Effects of preterm delivery on miRNAs regulating genes involved in the glycolytic pathway and glucose metabolism, as well as obesity-related genes, were also found, possibly indicating alterations in growth and metabolism of preterm infants [11]. It should also be emphasized that miRNAs are likely involved in mammary signaling during lactation, as distinct differences in many miRNAs were found when transitioning from lactogenesis to galactopoiesis and involution in dairy cows [25]. We also examined miRNAs from mothers delivering preterm infants and found that they also survive in vitro digestion and are taken up by human intestinal cells in culture [26]. We identified 330 miRNAs as preterm milk exosome miRNAs and the most abundant of these are similar to those found in term milk. Twenty-one low abundance miRNAs are specifically expressed in preterm milk exosomes compared to early term milk, possibly suggesting specific functions in the preterm infant.

Human milk cells also contain miRNAs, which have been extensively characterized [27]. The most highly expressed miRNAs were found to be key regula-
tors of milk components and may therefore be involved in the biosynthetic pathways in the mammary gland. The authors also found miRNAs targeting genes involved in body fluid balance, thirst, appetite, immune responses, and development, possibly affecting the recipient infant and complementing the miRNAs provided by exosomes.

Finally, miRNAs are found in milk fat, whey, exosomes, and cells, and their composition varies among these compartments [9, 28, 29]. Interestingly, a maternal high-fat diet was found to affect miRNAs in milk fat globules [30], suggesting that effects of the diet of the mother should be studied in more detail. Further investigations of the composition, digestibility, and cellular uptake/transfer, and effects of miRNAs in milk on different target cells should increase our understanding of their biological functions.

Conclusions

Human milk provides a rich source of miRNAs contained in exosomes and cells. The major miRNAs appear to be conserved among women and may therefore have biological significance for breastfed infants. These packaged miRNAs seem to survive digestion and are taken up by small intestinal cells, where they either can affect gene transcription locally or be transported by the systemic circulation to other target organs. Genes targeted by human milk miRNAs affect many pathways, in particular carbohydrate and energy metabolism, immune function, and brain development. Thus, breast milk may transfer genetic material to the infant and thereby affect gene transcription and regulation of cellular events in several tissues, possibly resulting in improved short- and long-term outcomes.

Disclosure Statement

Nothing to disclose.

References


Abstract
Human milk (HM) contains hundreds of proteins with very diverse functions that likely contribute to the short- and long-term beneficial effects of breastfeeding. These functions include serving as a source of amino acids, improving the bioavailability of micronutrients, including vitamins, minerals, and trace elements, providing immunologic defense, stimulating intestinal growth and maturation, shaping the microbiome, and enhancing learning and memory. Human milk proteins can be broadly classified into 3 categories: caseins, whey proteins, and mucins, which are present in the milk fat globule membrane. HM is whey predominant; however, the whey/casein ratio of HM changes from 90/10 in colostrum to 60/40 in mature HM. The whey proteins present in significant quantities in the whey fraction are α-lactalbumin, lactoferrin, IgA, osteopontin, and lysozyme. Additionally, bioactive peptides are formed during digestion of casein and whey, and glycans from glycoproteins are bifidogenic, adding further complexity to the functional properties of HM proteins. Recent advances in dairy technology have enabled isolation of bioactive milk proteins from bovine milk in sufficient quantities for clinical studies and, in some cases, addition to commercially available infant formula. Herein, the current evidence on HM protein composition and bioactivity of HM proteins is reviewed.

Introduction
Human milk (HM) provides nutrients in readily bioavailable forms that ensure optimal infant growth and development [1]. HM also contains a variety of bioactive proteins, lipids, and oligosaccharides that serve nonnutritional roles [2].
Over the past 30 years, infant formula composition has evolved to more closely mimic that of HM [3]. However, despite these attempts, disparities in growth [4], neurodevelopment [5], microbiome composition [6], immune function [7], and infectious disease incidence [8] persist between breast- and formula-fed infants. It is thought that the bioactive components in HM are in part responsible for these developmental differences. Herein, HM proteins, which provide indispensable amino acids for growth as well as bioactive proteins and peptides that serve nonnutritional roles in the developing neonate, will be reviewed [9].

**Human Milk Protein Composition**

The estimated 400 HM proteins [10, 11] are classified into 3 categories: caseins, whey proteins and mucins, the latter of which are present in the milk fat globule membrane (MFGM) [12]. Bovine milk is casein predominant, whereas HM is whey predominant. The HM whey/casein ratio changes over the course of lactation, declining from 90/10 in colostrum (days [d] 0–5) to 65/35 in transitional milk (d6–15), then 60/40 by 1 month postpartum and throughout the first year of lactation [12]. Bovine milk contains α-, β-, γ-, and κ-caseins, whereas HM contains β- and κ-casein, with lower concentrations of α-casein (Fig. 1). The

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Fig. 1. Comparison of the whey/casein ratios of mature human milk, bovine milk, and standard commercial infant formulas.
whey/casein ratio in formula is similar to mature HM (60/40), but formula contains all bovine milk caseins (Fig. 1). The concentrations of total and β- and κ-casein slightly rise between early and transitional milk, before declining and remaining relatively stable in mature milk. In contrast, the concentration of α-casein is constant throughout lactation (Table 1) [13].

The HM whey proteins present in highest concentration are α-lactalbumin, lactoferrin (LF), secretory IgA, osteopontin (OPN), and lysozyme (Table 2) [9, 11, 14]. In general, concentrations of these proteins sharply decrease from colostrum to mature HM, with the exception of lysozyme, which remains relatively steady [9]. Proteomic analysis of HM whey identified 115 unique proteins, of which 35% were related to immune response [15]. Other key functions were cell communication (17% of proteins), metabolism/energy production (16%), and general transport (12%) [15]. Proteomic analysis of

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Table 1. Concentrations (g/L) of total casein and casein subunits in human milk over the first year of lactation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Stage of lactation (days postpartum)</th>
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<tbody>
<tr>
<td></td>
<td>early (0–10)</td>
<td>transitional (11–30)</td>
<td>mature (31–365)</td>
<td></td>
</tr>
<tr>
<td>Total casein</td>
<td>2.49±0.41</td>
<td>2.59±0.59</td>
<td>1.92±0.72</td>
<td></td>
</tr>
<tr>
<td>α-Casein</td>
<td>0.34±0.09</td>
<td>0.33±0.07</td>
<td>0.33±0.18</td>
<td></td>
</tr>
<tr>
<td>β-Casein</td>
<td>1.29±0.28</td>
<td>1.46±0.46</td>
<td>1.03±0.53</td>
<td></td>
</tr>
<tr>
<td>κ-Casein</td>
<td>0.86±0.09</td>
<td>0.80±0.10</td>
<td>0.55±0.05</td>
<td></td>
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</tbody>
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Data are expressed as means ± SD. Adapted from Liao et al. -.

Table 2. Concentrations (g/L) of total protein and major whey proteins in human milk over the first year of lactation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Stage of lactation (days postpartum)</th>
<th></th>
<th></th>
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</thead>
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<tr>
<td>Total protein</td>
<td>20.6</td>
<td>15.7</td>
<td>14.8</td>
<td>11.1</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>4.56±0.41</td>
<td>4.3±0.41</td>
<td>3.52±0.27</td>
<td>2.85±0.24</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>6.15±0.89</td>
<td>3.65±1.19</td>
<td>2.46±0.27</td>
<td>1.76±0.28</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.32±0.01</td>
<td>0.30±0.01</td>
<td>0.28±0.11</td>
<td>0.38±0.15</td>
</tr>
<tr>
<td>Secretory IgA</td>
<td>5.45±1.7</td>
<td>1.5±0.22</td>
<td>1.10±0.32</td>
<td>1.14±0.21</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>0.180±0.10</td>
<td></td>
<td></td>
<td>0.138±0.09</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. Adapted from Haschke et al. [11] and Lönnerdal et al. [12].
The MFGM identified 191 proteins, with functions enriched in metabolism/energy production (21%), cell communication (19%), and general transport (16%), and to a lesser degree immune response (20%) compared to whey proteins [15, 16]. This paper identified many new MFGM proteins and provided insight into potential significance of MFGM for infant nutrition [16].

The predominant whey protein in bovine milk is β-lactoglobulin, which is not present in HM. Bovine milk also contains less α-lactalbumin, LF, and OPN than HM, with LF and OPN concentrations being 5 and 13% of HM concentrations, respectively [3, 17, 18]. Advances in dairy technology have enabled the isolation of bioactive milk proteins from bovine milk in sufficient quantities for clinical studies and, in some cases, addition to infant formulas [3, 19].

**Biological Activities of HM Proteins**

HM proteins exert a range of functions including: serving as a source of amino acids; improving micronutrient bioavailability; stimulating intestinal growth and maturation; supporting immunologic defense; shaping the microbiome; and enhancing learning and memory (Fig. 2). Some HM proteins exert activities across several categories (Table 3). For example, LF has been implicated as a nutrient transporter, in host defense, and for promoting intestinal, cognitive, and immune functions [18, 20–22] (Table 3). Additionally, bioactive peptides are

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**Fig. 2. Biological functions of human milk proteins.**
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formed during digestion of casein and whey proteins [12], and glycans released from HM glycoproteins by microbial glycosidases are bifidogenic [23], adding further complexity to the functional properties of HM proteins. Due to their multifunctional roles, 3 bioactive proteins, LF, OPN, and MFGM, have been isolated from bovine milk and tested for bioactivity in preclinical studies and human clinical trials [18].

Table 3. Human milk proteins associated with biological functions

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Protein</th>
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| Nutrition           | α-Lactalbumin  
|                     | α-Casein  
|                     | β-Casein  
|                     | κ-Casein  |
| Nutrient digestion and absorption | α1-Antitrypsin  
| Enzymes             | Amylase  
|                     | Bile-salt-stimulated lipase  
| Nutrient carrier proteins | α-Lactalbumin (calcium, zinc)  
|                     | β-Casein (calcium, phosphorus)  
|                     | Folate-binding protein (folate)  
|                     | Haptocorrin (vitamin B12)  
|                     | Lactoferrin (iron)  |
| Intestinal development | Growth factors (e.g., insulin-like growth factor-I, epidermal growth factor)  
|                     | Lactoferrin  |
| Host defense        | α-Lactalbumin  
|                     | Cytokines  
|                     | Haptocorrin  
|                     | κ-Casein  
|                     | Lactadherin  
|                     | Lactoferrin  
|                     | Lactoperoxidase  
|                     | Lysozyme  
|                     | Osteopontin  
|                     | Secretory IgA  |
| Prebiotics           | α-Lactalbumin  
|                     | Lactoferrin  
|                     | Milk glycans  
|                     | MFGM  |
| Cognition            | Lactoferrin  
|                     | MFGM  |

Adapted from Ballard and Morrow [2]; Haschke et al. [11]; Demmelmair et al. [18]; Donovan [21]; Karav et al. [23]. MFGM, milk fat globule membrane.
Bioactivities of Lactoferrin

LF is a nonheme iron binding protein that is one of the most widely studied HM proteins [20]. Randomized controlled clinical trials (RCTs) have supported antimicrobial and immunomodulatory activities of LF. For example, feeding 1.0 g/day of bovine LF for 9 months reduced *Giardia lamblia* colonization and increased growth of 12- to 36-month-old infants in Peru [24]. The addition of recombinant human LF (1.0 g/L) and lysozyme (0.2 g/L) to an oral rehydration solution reduced the duration of diarrhea in 5- to 35-month-old Peruvian infants hospitalized for diarrhea [25]. A Cochrane review concluded that “evidence of moderate to low quality suggests that oral LF prophylaxis with or without probiotics decreases late-onset sepsis and necrotizing enterocolitis stage II or greater in preterm infants without adverse effects” [26]. Thus, dietary LF has efficacy in both preventing and treating infectious diseases.

Orally administered LF exerts antibacterial and antiviral activities in the intestine through direct effects on pathogens and by affecting gastrointestinal and immune function [20, 21]. The latter functions are mediated by LF being taken up by cells via receptor-mediated pathways and affecting gene transcription [27]. In piglets, dietary bovine LF (1.0 or 3.6 g/L) increased intestinal cell proliferation, crypt depth, and β-catenin expression threefold [reviewed in 21]. Also, in piglets, dietary bovine LF modulated both systemic and intestinal immune development by stimulating a balanced T-helper-1/T-helper-2 cytokine response. Further, immune cells from piglets fed LF secreted more anti-inflammatory cytokines in an unstimulated state, while being primed for more a robust proinflammatory response when presented with a bacterial trigger ex vivo [reviewed in 21].

In terms of cognitive development, piglets fed bovine LF (0.6 g/L) from d3–d38 postpartum exhibited improved learning and memory in an 8-arm radial maze test compared with piglets fed an unsupplemented formula [22]. Moreover, ingested LF was associated with differential expression of 10 genes involved in the brain-derived neurotrophin factor (BDNF) signaling pathway in the hippocampus and upregulated the expression of polysialic acid, a marker of neuroplasticity, cell migration, and differentiation of progenitor cells, and the growth and targeting of axons, and increased phosphorylation of the cyclic adenosine monophosphate response element-binding protein, CREB, a downstream target of the BDNF signaling pathway, and an important protein in neurodevelopment and cognition [22]. Taken together, feeding LF at HM concentrations was bioactive with no reported adverse effects. LF is currently being supplemented to some commercial infant formulas; however, there are likely untapped potentials for LF to ad-
address some of the current immune, health, and cognitive differences between breast- and formula-fed infants; however, additional clinical trials are needed.

**Bioactivities of Osteopontin**

OPN is an acidic, glycosylated, and highly phosphorylated protein. It interacts with cell surface integrins and the CD44 receptor to influence biomineralization, tissue remodeling, and immune regulation [14, 18]. Feeding a formula with bovine OPN at the mean HM concentration to rhesus monkeys affected the expression of ∼2,000 intestinal genes and shifted the overall pattern of expression to be more similar to breastfed monkeys [28]. OPN influenced genes related to cell proliferation, migration, communication, and survival, and genes in pathways downstream from integrin and CD44 receptors [28]. In a recent RCT, formulas with either 65 or 130 mg bovine OPN/L was well tolerated and supported normal growth. However, infants consuming bovine OPN had a lower incidence of fever than standard formula and similar to breastfed controls [29]. Both OPN-supplemented infants had lower levels of TNF-α, higher levels of interleukin-2 and higher proportions of CD3+CD45+ T cells compared to the standard formula group [29, 30]. These studies indicate that oral OPN beneficially affects neonatal intestinal and immune development.

**Bioactivities of MFGM**

MFGM is the triple membrane system that encapsulates milk fat [31]. It contains cholesterol, glycerol-phospholipids, sphingolipids, and proteins, including mucin-1, butyrophilin, CD36, adipophilin, and lactadherin, which contribute to the antiviral and antibacterial activities of MGFM [31]. Indeed, an observational study of infants during the first 6 months of life found that rotavirus infection was negatively associated with the amount of HM lactadherin consumed, while intake of mucin and secretory IgA with milk was unrelated [32]. A recent RCT tested the safety and efficacy of MFGM in term infants randomized before the age of 2 months to a formula supplemented with a protein-rich MFGM preparation (4% of total protein) or a standard formula [reviewed in 31]. Formulas were fed until 6 months of age, the infants were followed until 12 months, and they were compared with a breastfed reference group. MFGM reduced diarrhea, otitis media, fever, and pyretic use [reviewed in 31]. Interestingly, *Moraxella catarrhalis*, a microbe commonly found in the middle ear in otitis media, was less abundant in the saliva of infants fed formula with MFGM, providing a potential mechanism of action [32]. In addition, the MFGM-supplemented group (105.8 ± 9.2) had significantly higher mean (± SD) scores in the cognitive domain of the Bayley Scales of Infant and Toddler Development compared to the
standard formula group (101.8 ± 8.0) [reviewed in 31]. Notably, the MFGM-supplemented formula-fed infants achieved cognitive scores not different than the breastfed infants (106.4 ± 9.5). Taken together, these studies establish the multifunctional actions of a single ingredient (OPN) in reducing the differences in cognitive and immune outcomes between breast- and formula-fed infants.

Future Directions

HM or formula provides the sole source nutrition of the first 6 months of life, which is a critical period of infant growth and development [1, 8]. Recent RCTs have shown that the bioactive proteins, LF, OPN, and MFGM, isolated from bovine milk, have beneficial effects on immune and cognitive outcomes in healthy term infants in the short term [31]. Future studies should investigate combinations of bioactive components (these HM proteins and other components, such as HM oligosaccharides or lipids). In addition, potential effects on longer-term programming of the immune system and cognitive functions and health outcomes must be investigated by following these infants in these study cohorts into later childhood.

Disclosure Statement

Sharon Donovan received financial compensation from the Nestle Nutrition Institute to co-chair the conference and prepare this chapter. Nestle Nutrition Institute had no input into the final content of the chapter.

References

28 Donovan SM, Monaco MH, Drnevich J, et al: Bovine osteopontin modifies the intestinal transcriptome of formula-fed infant rhesus monkeys to be more similar to those that were breastfed. J Nutr 2014;144:1910–1919.
In this session, new data on concentrations of micronutrients in human milk and factors affecting their concentrations were presented. Further, the physiological significance of bioactive factors in human milk such as proteins, oligosaccharides, microRNAs, and milk fat globule membranes (MFGM) was discussed and also highlighted by clinical studies in which breastfed infants were compared with infants fed formula with various sources of bioactive components.

Olle Hernell presented the various components of the human MFGM fraction, such as phospholipids, sphingomyelins and gangliosides, and membrane proteins like mucins, butyrophilin and lactadherin. MFGM is also a good source of sialic acid as part of gangliosides and glycosylated proteins. These components have been implicated in brain development and in the defense against infections. He then proceeded to present clinical trials in infants and children in which biological effects of MFGM or similar components have been evaluated. A few studies on infants fed formula with MFGM are encouraging and show positive effects on neurodevelopment and defense against infections, but further studies are needed to confirm this.

Norbert Sprenger explained how human milk oligosaccharides (HMOs) are synthesized in the mammary gland and how polymorphism in genes involved as well as stage of lactation can affect their composition in breast milk. Several observational and basic research studies strongly suggest that HMOs influence early-life microbiota and mucosal immunity and inhibit pathogens, thereby contributing to protection against infections. He presented the few clinical trials
that have been performed on infants fed formula with added simple HMOs, such as 2′-fucosyllactose and lacto-N-neotetraose. The studies were largely designed with growth and tolerance as primary outcomes, but results suggested lower respiratory tract illness and reduced need for antibiotics as well as a shift in early-life microbiota composition towards that of breastfed infants.

Ardythe Morrow discussed how diet and genetics can affect fatty acids and fat-soluble vitamins in human milk. She presented results from a three-country study particularly focusing on long-chain polyunsaturated fatty acids and branched chain fatty acids and showed how maternal dietary intake can affect their milk concentrations. Consequences of mothers carrying different fatty acid desaturase genes were also discussed. Physiological consequences of deficiencies of the fat-soluble vitamins (A, D, E, and K) were presented as well as approaches to increase milk concentrations of these vitamins by supplementation or dietary modifications.

Lindsay Allen discussed how recent developments in analytical methods used to determine water-soluble vitamins in human milk have advanced our knowledge of these micronutrients. Although there are few changes in concentrations of water-soluble vitamins during a feed or diurnally, factors such as duration of lactation and maternal supplementation do affect their concentrations in breast milk. Some of these vitamins are also affected by parity, maternal illness, and preterm delivery. She presented the various forms of these vitamins that can be found in human milk and also reported on recent multi-country studies on water-soluble vitamins in breast milk. Lindsay Allen also emphasized that data are still inadequate to properly set AIs (adequate intakes) and urged the need for more studies to facilitate the establishment of well-founded reference values.

Bo Lönnerdal introduced the concept of microRNAs and how they are carried by exosomes in breast milk. These microRNAs have the capacity to bind to cellular mRNA and affect gene expression. He then described microRNAs that have been found in human milk, how they vary during lactation and how they can resist digestion in vitro under conditions that mimic those of the infant gastrointestinal tract. Following digestion with pepsin at low pH and exposure to pancreatic enzymes at neutral pH, microRNAs in milk exosomes were not only found to be remarkably stable, but were taken up by human intestinal cells in culture. Thus, it is possible that microRNAs in human milk can be taken up by the infant intestinal epithelium and affect gene transcription locally or be transferred to the systemic circulation and affect gene expression in other tissues.

Sharon Donovan reviewed the protein composition of human milk, including the three major fractions, caseins, whey proteins, and mucins. Many of these proteins have physiological activities, and since some of their bovine counterparts are available commercially, they can be added to infant formula. She then
presented studies in piglets and in infants that have evaluated the effects of adding lactoferrin, osteopontin, and MFGM to formula. In piglets, lactoferrin was found to stimulate intestinal development and modulate immune function, and also affect neurodevelopment. Osteopontin was found to affect gene expression in the small intestine of infant rhesus monkeys and make it more similar to that of breastfed infants, and a clinical trial of formula with added osteopontin showed reduced illness and a positive effect on serum cytokines. A study on human infants fed formula with MFGM showed less illness and improved cognitive development. Thus, there is support for these components reducing infections and affecting immune function, and possibly also brain development, but further studies in both preterm and term infants are needed.

Bo Lönnerdal
Clinical Aspects of Human Milk on Infant Health Outcomes

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Abstract

It is well established that nutrition during the first 1,000 days of life can have a long-term effect on growth, metabolic outcome, and long-term health. We review the long-term anthropometric follow-ups of children with risk of later morbidity: (a) very-low-birth-weight (VLBW) infants who have birth weights <10th percentile of weight and receive fortified breast milk, (b) infants from developing countries who are breastfed according to the present recommendations but have low birth weight and length, and (c) children from developed countries who were enrolled in randomized controlled trials (RCTs) to test if breastfeeding and low-protein formulas can prevent from rapid weight gain and childhood obesity. VLBW infants can be appropriate, small for gestational age (SGA), or intrauterine growth retarded (IUGR). SGA and IUGR (due to placenta insufficiency) infants are born with birth weights <10th percentile of weight for gestational age (GA). We provided fortified breast milk until 52 weeks of GA to 31 SGA and 127 IUGR infants and followed up growth until 24 months. IUGR infants showed lower weight gain between birth and 3 months and had lower weight between 3 and 24 months (p < 0.05; ANCOVA). No significant BMI differences between SGA and IUGR infants were observed. It seems that IUGR infants receiving fortified breast milk need special attention, because without further improvement in breast milk fortification weight gain after discharge from hospital might be too slow. In developing countries, length and weight of breastfed infants during the first 2 years are strongly influenced by the respective anthropometric parameters at birth. Studies in the Gambia and Zimbabwe indicate that only breastfed infants with birth length and weight above the respective WHO 0 z-scores continue with adequate growth and have length and weight above the WHO 0 z-scores at 18 and 24 months. Prevalence of stunting and wasting in the overall Gambia breastfed infant population rapidly increases during the first year, peaks at around 3 years, but decreases thereafter. Long-term

Early-Life Nutrition, Growth Trajectories, and Long-Term Outcome

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Abstract

It is well established that nutrition during the first 1,000 days of life can have a long-term effect on growth, metabolic outcome, and long-term health. We review the long-term anthropometric follow-ups of children with risk of later morbidity: (a) very-low-birth-weight (VLBW) infants who have birth weights <10th percentile of weight and receive fortified breast milk, (b) infants from developing countries who are breastfed according to the present recommendations but have low birth weight and length, and (c) children from developed countries who were enrolled in randomized controlled trials (RCTs) to test if breastfeeding and low-protein formulas can prevent from rapid weight gain and childhood obesity. VLBW infants can be appropriate, small for gestational age (SGA), or intrauterine growth retarded (IUGR). SGA and IUGR (due to placenta insufficiency) infants are born with birth weights <10th percentile of weight for gestational age (GA). We provided fortified breast milk until 52 weeks of GA to 31 SGA and 127 IUGR infants and followed up growth until 24 months. IUGR infants showed lower weight gain between birth and 3 months and had lower weight between 3 and 24 months (p < 0.05; ANCOVA). No significant BMI differences between SGA and IUGR infants were observed. It seems that IUGR infants receiving fortified breast milk need special attention, because without further improvement in breast milk fortification weight gain after discharge from hospital might be too slow. In developing countries, length and weight of breastfed infants during the first 2 years are strongly influenced by the respective anthropometric parameters at birth. Studies in the Gambia and Zimbabwe indicate that only breastfed infants with birth length and weight above the respective WHO 0 z-scores continue with adequate growth and have length and weight above the WHO 0 z-scores at 18 and 24 months. Prevalence of stunting and wasting in the overall Gambia breastfed infant population rapidly increases during the first year, peaks at around 3 years, but decreases thereafter. Long-term
growth trajectories indicate later start of puberty and slow pubertal growth, but adult weight and height are not reached before 20–24 years. In adulthood, prevalence of stunting and wasting is much lower than during any period of childhood. Maternal risk factors, such as childhood marriage and poor nutrition before and during pregnancy, need to come into focus to improve birth length and weight and lower high stunting rates. Term breastfed infants from overweight/obese mothers and breastfed infants with rapid weight gain during infancy have increased risk of childhood obesity. Infants who are exclusively breastfed 4–6 months or receive low protein follow-up formulas (high-quality protein) grow slower during the first 2–3 years than infants fed high-protein formulas. During follow-up examinations at 5–6 years, they have lower BMI and obesity prevalence. Body composition measurements (DEXA) at 5–8 years in children who were breastfed and received low- or high-protein formula during infancy indicate that breastfeeding and feeding low-protein formulas are associated with lower gain of fat mass. Longitudinal cohort studies show that high-protein intake during the first 2 years results in higher BMI at 9 years and during adulthood. The studies presented indicate that breastfeeding but also other pre- and postnatal nutritional, epigenetic, and environmental factors influence growth trajectories and long-term health.

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Introduction

It is well documented that maternal factors, events during the first 1,000 days of life, and early nutrition can have a long-term effect on growth, metabolic outcome, and long-term health [1, 2] (www.thousanddays.org). Growth of very-low-birth-weight (VLBW) infants (birth weight <1,500 g; gestational age [GA] <32 weeks) must be carefully monitored to avoid low postnatal weight gain that can be associated with a poor neurodevelopmental outcome [3, 4]. Faster weight gain on the other hand – the upward percentile crossing on a weight chart [5–7] – can be associated with childhood obesity and impaired metabolic outcome. We present growth trajectories of 2 cohorts of high-risk VLBW infants with birth weights <10th percentile for gestational age (GA) who received fortified breast milk until 52 weeks of GA.

Reviews of cross-sectional data from developing countries indicate that exclusive breastfeeding until 6 months helps to prevent from stunting, wasting, as well as from increased morbidity and mortality [8]. However, influences of maternal and intrauterine nutrition and complementary feedings that can be important for long-term growth and health have not been properly addressed. A long-term study in the Gambia [9] indicates that there might be windows of opportunity for nutritional intervention before and during pregnancy and after the first 1,000 days.

Breastfed infants in developed countries receive less protein than infants fed traditional infant/follow-up formulas [10]. Clinical trials indicate lower weight
and body fatness in infants with lower protein intake [6, 11–13]. Long-term follow-up shows that higher protein intake during the first 12 months results in increased risk of overweight and obesity later in life [14]. To better understand long-term effects of early protein intake, we review indicators of body fatness and body composition in 5- to 8-year-old children who were breastfed or received formulas with different protein content.

**Very-Low-Birth-Weight Infants**

International pediatric societies still argue that postnatal growth of VLBW infants should be close to that of the in utero growing fetus. However, during postnatal adaption, extracellular water space is contracting, which results in 7–11% weight loss [15]; risks of morbidity and intolerance of enteral feeding are high, and neonatal intensive care (NICU) staff sometimes has to limit nutrient intake. VLBW infants (<1,500 g) reach their lowest weight between the 4th and 6th day of life, and birth weight is regained between 13 and 18 days of postnatal life [15]. After postnatal adaption, the new growth trajectories are parallel but approximately \(-0.8\) z-scores below fetal growth standards [15].

Postnatal weight trajectories of VLBW infants who are born <10th percentile of weight for GA (“born too small”) are not so clear. For those VLBW infants, the terms small for GA (SGA) and intrauterine growth restriction (IUGR) are often synonymously used [16]. However, the underlying conditions that limit intrauterine growth are different: SGA represents a constitutionally small VLBW infant [17]. IUGR is a VLBW infant who fails to reach its potential growth due to maternal placental insufficiency, resulting in an impaired placental nutrient transport [18]. IUGR is a prenatal diagnosis, based on pathological ultrasound criteria according to the Clinical Guidelines of the Society of Maternal-Fetal Medicine [19]. In neonatal intensive care and during follow-up, all growth-restricted VLBW infants are treated the same, which includes nutritional management after birth: ESPGHAN recommends to apply an “enhanced nutrient strategy” after discharge from hospital up to the 52nd week of GA to all growth-restricted VLBW infants – preferably fortified human milk – regardless if they are SGA or IUGR [20]. Actually, reliable data proving that the recommended “enhanced nutrient strategy” is safe and effective both for VLBW infants who are SGA or IUGR are lacking, because most studies combined both groups in follow-up on growth. Epidemiological studies indicate that early nutrition during the pre- and postnatal life can have an impact on long-term health: undernutrition in utero permanently changes the body’s structure, function, and metabolism in ways that lead to obesity, insulin resis-
tance, diabetes type 2, atherosclerosis, and associated cardiovascular ill-health later in life [1, 2, 21]. Postnatal centile crossing defined in terms of an upward change in weight z-score during 1 month [5–7; “rapid catchup growth”] in malnourished newborns can be associated with obesity and cardiovascular diseases in later life [22, 23]. In particular, it is not clear how IUGR infants react if they are exposed to prolonged postnatal exposure to an “enhanced nutrient concept.” Postnatal nutrition of all segments of VLBW infants must be safe, help to achieve optimal growth but also minimize the potential risk for later ill-health [23]. All VLBW infants in our NICU receive fortified breast milk until 52 weeks of corrected GA according to the present recommendations [20]. Long-term follow-up of postnatal growth of those infants is of interest to document their growth and development beyond infancy.

We investigated the impact of the “enhanced nutrient strategy” [18; ESPGHAN] on anthropometric parameters of all SGA and IUGR VLBW infants who were treated in our NICU during the last decade and could be followed up until 2 years in our outpatient clinic. All study infants had pre- and postnatal examinations in regular intervals: 31 SGA infants without genetic defects or malformations and 127 IUGR infants with intrauterine pathological ultrasound pattern [19]. Median birth weights of SGA and IUGR were 600 and 688 g (nonsignificant), and median GAs were 25 weeks (+6 days) and 29 weeks (+1 day; \( p < 0.001 \)), respectively [24]. Enteral feedings of all infants started with breast milk that was then fortified with a human milk fortifier to increase protein, caloric, and micronutrient concentrations of feedings. At discharge, 68% of the infants exclusively received fortified breast milk [24].

Weight gain of IUGR infants was lower between birth and 3 months with a change from –1 to –2 WHO z-scores (Fig. 1). Poor weight gain was mainly observed after discharge from hospital. This resulted in significantly lower weight of IUGR infants between 3 and 24 months (corrected for sex and GA; ANCOVA). SGA and IUGR infants gained 1.3 and 1.7 z-score points between 3 and 24 months corrected for GA. Mean weight of SGA infants crossed the 10th percentile of the WHO standards (http://www.who.int/childgrowth/standards/en/) at 6 months, whereas weight of IUGR infants remained below the 10th percentile until 24 months. Standard deviations indicate that until 24 months some infants would be still classified as malnourished, because their weight was below –2 z-scores of the WHO charts. Both groups did not show upwards crossing of percentiles during 1 month [7], that is, no accelerated weight gain until 24 months.

BMI of both groups did not deteriorate between birth and 3 months. Between 3 and 24 months, no significant BMI differences between SGA and IUGR infants
were observed. SGA and IUGR infants gained 0.6 and 0.8 z-score points between 3 and 24 months (Fig. 2).

Our preliminary data indicate lower weight gain of IUGR infants during the period when the enhanced nutrient strategy [20] is applied. This results in lower weight until 24 months. To answer the question if there is a need to develop separate nutrition guidelines for VLBW infants who are SGA or IUGR, randomized controlled studies (RCTs) with further improved breast milk fortification which include anthropometry, body composition, epigenetic, metabolic, and neurodevelopmental outcomes, and the microbiome are necessary.

Fig. 1. Weight z-scores (x, SD) of SGA- and IUGR VLBW infants at birth and from 3 to 24 months (corrected for prematurity). Weight reference at birth: http://www.merckmanuals.com/en-ca/professional/pediatrics/perinatal-prob. Weight standard from 3 to 24 months: http://www.who.int/childgrowth/standards/en/. Analysis: ANCOVA with birth-weight and sex as covariates. *p < 0.05.
Before a child’s 6th birthday, the brain matures more rapidly than at any other time in life. Poor nutrition can have a profound, lifelong impact on a child’s growth, learning, and health. In 2018, WHO, UNICEF, and the World Bank (WHO/UNICEF) report that the number of children (<5 years) who are stunted has worldwide declined from approximately 250 million (1990) to 150 million (2015), but the number of stunted children increased in sub-Saharan Africa from 45 to 57 million in 2015. Therefore, it seems unlikely that the global WHO targets 2025 to reduce stunting by 40% will be met in sub-Saharan Africa during the next decade. Exclusive breastfeeding plays a key role in the prevention of stunting and wasting: the associations of breastfeeding with growth and health in developing countries can be studied by repeatedly analyzing demographic health surveys (DHS; US) data sets that provide information on nutrition, growth, and health. We reviewed data of more than 130,000 infants and small children (0–6, 6–12, and 12–24 months) from 20 developing countries that were collected at least twice in intervals of 5–10 years during the last 2 decades [8; unpubl. data]. Our sample (Table 1) indicates an exclusive breastfeeding rate of 32% (96% overall breastfeeding rate) during the first 6 months and is therefore representative for all developing countries, where WHO estimates the respective exclusive breastfeeding rate to be about 34%.
Table 1. Comparison of growth and health of infants (0–6 months) from 20 developing countries

<table>
<thead>
<tr>
<th></th>
<th>Stunting (10%)</th>
<th>Wasting (6%)</th>
<th>Diarrhea</th>
<th>Fever</th>
<th>Cough</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>–2%</td>
<td>±0%</td>
<td>–6%</td>
<td>–9%</td>
<td>–9%</td>
</tr>
<tr>
<td>NO</td>
<td>–9%</td>
<td>–4%</td>
<td>–10%</td>
<td>–12%</td>
<td>–9%</td>
</tr>
</tbody>
</table>

Exclusive breastfeeding (32%) vs. nonexclusive breastfeeding (NE; 64%) and no breastfeeding (NO; 45%); *p < 0.05.

Table 1 shows growth and health of infants who are exclusively or not exclusively breastfed, or not breastfed. Prevalence of stunting and wasting was already high during the first 6 months. Exclusive breastfeeding was associated with significantly higher weight, length, and lower probability of stunting, wasting, and infections. Between 6 and 12 months when 90% of infants were still breastfed and stunting and wasting prevalence was already 24 and 11%, respectively; the association of breastfeeding with lower prevalence of stunting and wasting was no more present, but the probability of infections tended to be lower [8]. Between 12 and 24 months, when stunting (45%) and wasting prevalence (14%) was very high, 74% of the infants still received breast milk. During the second year of life, breastfeeding was associated with significantly higher stunting and wasting prevalence [8]. DHS data are cross-sectional and indicate associations and no causal relationships. Like many other studies, they underline the importance of exclusive breastfeeding for growth and health during the first 6 months, but a recent well-controlled longitudinal cohort study in the Gambia (n = 756) challenges that exclusive breastfeeding improves length for age z-scores at 6, 12, and 24 months [25]. DHS data of infants between 6 and 24 months also reflect the influence of low-quality complementary feedings and poor environmental conditions in developing countries, which contribute to the high stunting and wasting rates. A systematic review of trials in developing countries which provide complementary foods and education [26] showed significant effect sizes of +0.17 for length/height for age z-scores and +0.35 for weight/age z-scores. Similar effects were found when energy density of the usual complementary foods was increased.

Victora et al. [27] showed that in poor regions of the world, such as Southeast Asia and Africa, growth is already disturbed during the embryonic and fetal period. Average height/age z-scores (HAZ) at birth were –0.75 and –0.35 and further declined during the first 2 years by approximately 1.5 HAZ. No detailed data on feeding mode were included. Growth trajectories from 2 well-controlled African cohorts [28; unpubl. data provided to the authors; 29, 30] with strong
breastfeeding support showed the importance of maternal stature, nutrition, and health, as well as maternal nutrition before conception and during pregnancy: growth trajectories of infants who were in the top 10th percentile segment of length at birth grew almost according to the WHO standards until 2 years. However, those infants in the bottom 10th percentile segment at birth (i.e., newborns with disturbed intrauterine growth) showed poor growth and had mean length at 2 years that was below the –2 z-score of the WHO standards. In addition to breastfeeding support, future key targets should be to improve nutrition of adolescent girls, young women, and during pregnancy, but studies with targeted interventions are still lacking [31–33].

Timing of cell-proliferative potential of organ systems across childhood and adolescence does not support the concept of a catchup window closing at 24 months [28]. Analysis of data from various sources shows that catchup in height z-scores occurs after 24 months in many poor populations, even in the absence of interventions: early height growth in 5 populations (Brazil, Guatemala, India, Philippines, and South Africa), each studied longitudinally and brought together in the COHORTS collaboration [34], confirmed the rapid fall-off in height z-scores between birth and 24 months in all 5 countries, but also showed significant regain between 24 and 48 months in 4 of the 5 cohorts. Both the falloff and catchup was observed irrespective of the final height attained. India was a distinct outlier with no signs of catchup. Prentice et al. [28] showed that in children from rural Gambia who were breastfed substantial height catch-up occurred between 24 months and childhood and again between childhood and adulthood, even in the absence of any interventions. Development of better adaptive response mechanisms against pathogens might reduce the frequency and severity of growth-limiting infections. Longitudinal growth data from that country illustrate that an extended pubertal growth phase allows very considerable height recovery, especially in girls during adolescence. In light of the critical importance of maternal stature to her children’s health, it becomes clear that adolescence represents an additional window of opportunity during which substantial life cycle and intergenerational effects can be accrued.

The traditional view has been that the “window of opportunities” for growth-and health-promoting interventions should the period between the day of conception and 24 months (“first 1,000 days”; www.thousanddays.org). Interventions before the first 1,000 days have only been recently in focus of scientific research [28, 31–34]. Epigenetically mediated early-life and/or intergenerational effects may also contribute to population diversity in later growth [28]. Height catchup in young children, even in the absence of external nutritional interventions, clearly contradicts the widely held impression that a window of opportunity closes at 24 months of age [9]. The extent of catchup after 24 months is
highly context specific and presumably reflects the availability of foods, food consumption patterns, the composition of diets, and the prevailing burden of infections (especially those affecting gastrointestinal function).

Is Low Protein Intake during the Breastfeeding Period and Beyond a Factor That Contributes to Obesity Prevention?

Term breastfed infants from overweight/obese mothers and breastfed infants with rapid weight gain during infancy have an increased risk of obesity during childhood, adolescence, and adulthood, which can be associated with disturbed metabolic outcome and higher risk of diabetes and cardiovascular disease [35–37]. Dewey at al. [38] compared weight, length, and weight-for-length ratios of infants who were breastfed or fed infant formulas and found that breastfed infants are leaner. Energy intake of breastfed infants at 3 and 6 months was 13–20% lower, but protein intake was 43–47% lower [39]. Formulas in the Darling study [39] had higher protein concentrations than modern formulas which have been on the markets during the last 10 years. It was suggested that higher protein intake could be the reason for “accelerated growth” in formula-fed infants [39], which could be a risk factor later in life. Differences in growth between breast- and formula-fed infants are one of the reasons why the WHO (http://www.who.int/childgrowth/standards/en/) started to collect data to construct the WHO growth standards, which are based on infants who were predominately breastfed at least for 4–6 months and where breastfeeding continued beyond 6 months. It must be pointed out that until now there are no randomized trials which compare long-term growth of exclusively breastfed (i.e., 4–6 months) and formula infants. For obvious reasons, it is most unlikely that such trials will be conducted in the future. PROBIT in Belarus [40] is the only randomized cluster intervention trial that looks at feeding modes during the first 6 months and long-term growth. A breastfeeding intervention group (modeled by the WHO/UNICEF baby-friendly hospital initiative) was compared with a group without any intervention. Exclusive breastfeeding rates at 3 months in the intervention and control groups were 43.3 and 6.4% ($p < 0.001$). Corresponding rates at 6 months were as low as 7.9 and 0.6%. No differences in growth and adiposity between groups were found at 6.5 and 11.5 years [41]. Because of the low percentages of breastfed infants in the intervention group at 3 and 6 months, the PROBIT study cannot answer the question if exclusive breastfeeding according to the international recommendations (4–6 months) helps to lower the prevalence of childhood obesity in developed countries.
RCTs that compare and follow up growth of appropriate- or SGA term newborns [6, 11–13] can help to answer the question: infants were exclusively breastfed for 3–6 months or received low- or high-protein formulas until 9 or 12 months of age. Caloric density of higher- and lower-protein formulas was almost equal in three trials [11–13], whereas in one trial the high-protein formula had a 6% higher energy density [6]. During and/or at the end of the intervention, all studies showed lower weight in the breastfed and low-protein formula groups than in the high-protein formula groups (Table 2) [11–13, 26]. Follow-up at 2 [11, 12] and 3 years [12] indicated lower weight and BMI in the breastfed and low-protein formula groups. The breastfed group in the multicenter US study [13] at 5 years still had lower weight (–853 g; 95% CI 12.9–1,695.0; $p \leq 0.047$) and BMI (–0.56; 95% CI 0.09–1.02; $p \leq 0.021$) than the high-protein formula group. Differences between the breastfed and low-protein formula group were small and not significant. At 6 years, the European multicenter CHOP study [11, 14] reported significantly lower BMI and lower percentage of obesity in the breastfed and the low- than in the high-protein formula group.

### Table 2. RCTs. Protein intake during infancy, growth, and body composition

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Exclusive BF</th>
<th>Study outcome</th>
<th>Follow-up, years</th>
<th>Body composition</th>
<th>Body composition, years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g protein/100 kcal</td>
<td>Δ weight</td>
<td>Δ BMI</td>
<td>Δ weight</td>
<td>Δ BMI</td>
</tr>
<tr>
<td></td>
<td>Duration of I</td>
<td>Weight</td>
<td>Follow-up, years</td>
<td>BMI</td>
<td>Method</td>
</tr>
<tr>
<td>[6]</td>
<td>BF: 0–3 months</td>
<td>LPIF: 2.05</td>
<td>HPIF: 2.5</td>
<td>I: 0–9 months</td>
<td>Weight: LPIF &lt; HPIF</td>
</tr>
<tr>
<td>[6]</td>
<td>LPIF: 2.1</td>
<td>HPIF: 2.7</td>
<td>I: 0–6 months</td>
<td>5–8 years BMI</td>
<td>NS</td>
</tr>
<tr>
<td>[13]</td>
<td>BF: 0–4/6 months</td>
<td>IF: 2.1 (both groups)</td>
<td>LPIF: 1.61</td>
<td>HPFF: 2.15</td>
<td>I: 3–12 months</td>
</tr>
<tr>
<td>[12]</td>
<td>BF: 0–4/6 months</td>
<td>IF: 1.8 (both groups)</td>
<td>LPIF: 1.65</td>
<td>HPFF: 2.7</td>
<td>I: 3–12 months</td>
</tr>
</tbody>
</table>
|      | BF, breastfeeding; I, intervention; LPIF, low-protein infant formula; HPIF, high-protein infant formula; IF, infant formula; LPFF, low-protein follow-up formula; HPFF, high-protein follow-up formula; NR, not reported.
**Body Composition**

A long-term follow-up of 4 RCTs with body composition measurements between 5 and 8 years has been published: Singhal et al. [6] compared fat mass (kg) of children with birth weights <10th percentile (weight) who were exclusively breastfed or received a low- or high-protein formula (Table 2). Unadjusted body fat mass was about 40 and 26% lower [6] in the breastfed and low-protein formula groups than in the high-protein formula group. Gender-adjusted differences between the low- and high-protein group were highly significant (p < 0.009). In another study [9], children with birth weights <20th percentile (weight) were fed a low- or high-protein formula until 6 months. The high-protein formula provided 12% more calories per 100 ml. No breastfed group was included. In the low-protein formula group, fat mass at 5–8 years was 18% lower. When confounders were considered, the difference became significant (p ≤ 0.04). In a subsample of a US multicenter RCT [13], longitudinal changes in body composition between 6 and 60 months of age have been documented. [37]. References [42] indicate that children during this period are becoming about 10% leaner. In the US study [13, 37], percent body fat decreased 7.3, 4.9, or 2.0%, in children who were exclusively breastfed or received low- or high-protein formula, respectively. Differences between the breastfed and low-protein formula group on the one hand and the higher-protein group on the other hand were statistically significant (p < 0.05) [37]. One RCT in children from overweight mothers [12, 37] did not show differences in body composition but had poor follow-up of DEXA measurements at 5 years (<30%).

**Cohort Studies**

Two longitudinal cohort studies support the findings from RCTs that low protein intake during early life with breast milk or low-protein formula is associated with a lower obesity risk later in life. Braun et al. [43] reported that in a Dutch cohort lower protein intake at 12 months was associated with lower weight and BMI at 9 years. In the same cohort, lower protein intake and slower weight gain during infancy was associated with lower fat mass (DEXA) and lower BMI at 6 years [44]. Rolland-Cachera [45] showed that in a cohort which represented the French childhood population, lower protein intake during the first 2 years was associated with significantly lower BMI at 20 years.

It is evident that low protein intake with breast milk can contribute to later obesity prevention. The effect size has been estimated to be as high as 20% [6]. RCTs show that low-protein formulas can contribute to obesity prevention if breastfeeding is not possible or too short. It seems that the second half of the first year and the second year are more critical because present follow-up formulas...
and cow’s milk provide 2.5 and 4 times more protein than breast milk [10].
EFSA, the European Food Safety Agency, recently approved follow-up formulas
with 1.6 g protein per 100 kcal if clinical trials prove safety.

Conclusions

VLBW infants who are IUGR show low weight gain after discharge from hospital
when they receive fortified breast milk. RCTs are necessary to confirm the results
of our cohort study and to test new fortification strategies of breast milk. Stunting
rates in children from developing countries are still high.Exclusive breastfeeding
is important to prevent infants from stunting. Further preventive measures in-
clude nutritional supplementation of young women before and during pregnancy,
promotion of breastfeeding and improvement of quality of complementary foods.
RCTs which include follow-up of growth and body composition during childhood
indicate that breastfeeding and the use of low-protein formulas can contribute to
the prevention of rapid weight gain during infancy and childhood obesity.

Disclosure Statement

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food companies (Hipp, Nestle, Kabrita) and receives honoraries for keynote lectures and
workshops on congresses or other scientific events (Baxter, Milupa, Hipp, Nestle).

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Early-Life Nutrition and Cognitive Development: Imaging Approaches

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Abstract

Brain development in the first years of life is the most dynamic and perhaps the most important phase of brain maturation. While it is widely recognized that nutrition plays a key role in early brain development, particular nutrients will most likely differentially affect distinct aspects of brain development. The critical dosage windows and time frames for various nutrients at different stages of brain development are likely dissimilar. Therefore, efforts have been devoted to identifying potential associations between nutrients and early brain development. However, behavioral assessments are typically employed as the outcome measures, which are known to suffer from low sensitivity and the inability to provide neural substrates underlying brain functional maturation. In contrast, magnetic resonance imaging is capable of providing detailed anatomical and functional information – an ideal tool to characterize brain functional development and nutrition. Our team has developed strategies that enable imaging of typically developing children from birth to teens without sedation. Quantitative assessments of brain structural and functional development during the first years of life have been accomplished, which reveal important features of early brain development. These developed tools will most likely substantially enhance our ability to rigorously characterize the interplay between nutrients and early brain development.

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Introduction

Brain development in the first years of life is the most dynamic and perhaps the most important phase of brain functional maturation [1]. Thus, efforts to shed light on the underlying maturation processes have been actively pursued [2–5]. Early brain development can potentially be categorized into 7 cellular stages encompassing both pre- and postnatal periods [6]. Neurogenesis represents the first stage of brain development, followed by cell migration (2nd stage). Once neurons arrive at their final destinations, cell differentiation occurs (3rd stage). While it has been shown that neurogenesis, cell migration, and cell differentiation are also present in a selected few brain regions postnatally [7], they predominately occur prior to birth. In contrast, the remaining 4 stages – dendrite and axonal growth, synaptogenesis, synaptic pruning, and myelination – mainly arise postnatally. These complex and dynamic cellular processes set the foundation for remarkable cognitive maturation processes during the first years of life. Basic sensory functions such as visual and auditory functions start to develop during the third trimester and continue throughout the first years of life [8]. Language functions emerge during the second half of the first year and continue to mature throughout the first several years of life. Although higher-order brain functions are known to mature later in life and follow a protracted developmental trajectory, primitive higher-order brain functional networks resembling those observed in adults have also been reported during the first year of life [9, 10]. In addition, joint attention emerges around 9–10 months [11], which includes a set of complex behaviors considered as necessary precursors for (1) subsequent social communication such as the acquisition of spoken language and (2) increasingly complex social-cognitive capacities such as Theory of Mind [12]. Working memory capacity rapidly increases during this interval [13], laying the foundation for the development of more complex executive functioning. Infants between 9 and 10 months of age also begin to demonstrate more specialized face-processing skills (e.g., losing the global ability to effectively/consistently discriminate faces from other species) and specialized language processing (e.g., losing the global ability to discriminate nonnative language phonemes). Although basic brain functions, such as fine motor skills and auditory function, continue to mature, emphases on maturation of higher-order brain functions emerge rapidly after the first years of life and persist to teens.

Any adverse effects leading to the deviation from these well-orchestrated processes could result in life-long impacts on the health and development of our brain. Therefore, it is not surprising that extensive efforts have been devoted to identifying critical factors that could influence maturation processes of brain
functions, including, but not limited to, environmental factors [14], parent-child interaction [15], and nutrition [16, 17]. In the context of this article, nutritional factors and their effects on normal brain functional development will be discussed. It is widely recognized that adequate nutrition is necessary for normal brain development during pregnancy and infancy, and particular nutrients most likely affect distinct aspects of brain development. However, detailed information on the critical dosage windows and time frames for various nutrients needed at different stages of brain development remains lacking. Therefore, approaches capable of assessing the outcomes of exposure to different nutrients, including their timing and dose, are greatly needed, enhancing our understanding of complex interactions between nutrition and brain development throughout early childhood. To this end, this article will first provide a brief overview of several key nutrients in early brain development, followed by how noninvasive imaging methods may provide means to characterize nutritional impacts (both in their timing and dose) on early brain developments, and, finally, a brief overview of the brain-gut axis.

Nutrition in Early Brain Development

Undoubtedly, nutrition is one of the key factors that could have lifelong impacts on brain cognitive development, particularly during the critical time periods of early brain development. Table 1 summarizes a list of key nutrients and their roles on different aspects of brain development. Speaking to the complexity of this relationship, each nutrient separately affects distinct aspects of brain development at different stages of brain development. For example, long-chain polyunsaturated fatty acids (LCPUFAs) are essential for both neurogenesis as well as synaptogenesis, affecting both pre- and postnatal brain development. Sphingomyelin is crucial for white-matter (WM) myelination, which undergoes rapid developmental processes from late third trimester throughout the first years of life. It should also be recognized that different nutrients could contribute to similar aspects of brain development, functioning in a complementary or synergistic manner. For example, iron, choline, as well as sphingomyelin have been implicated to play key roles in WM myelination. Given these complex interactions between nutrients and the variable paces of brain structural and functional development, identifying a direct causal relation between nutrients and early brain development has been extremely challenging. Nevertheless, general consensus of potential benefits on early brain development of several essential nutrients has been demonstrated, and a brief review of these key nutrients is provided below.
Iron, as an essential structural component of the hemoglobin molecule, plays a critical role in transporting oxygen throughout the body. The role of iron as a vital nutrient in brain development is highlighted by its sensitive dose-response window; both iron excess and deficiency may induce abnormal brain development. Iron deficiency alters myelination (WM), monoamine neurotransmitter synthesis (striatal-frontal), and neuronal and glial energy metabolism (hippocampal-frontal) [17]. These brain processes are correlated with developmental behaviors, such as speed of processing (myelination), changes in motor and affect (monoamines), and recognition memory (hippocampus). Infant iron deficiency anemia is a risk factor for both short- and long-term cognitive impairment. These effects may persist even if treatment is provided during infancy, highlighting the gravity of the perinatal role of iron. In multiple longitudinal

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Roles</th>
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<tbody>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>Myelination, neurotransmission, brain growth, cofactor for brain enzymes</td>
</tr>
<tr>
<td>Zinc</td>
<td>Neurogenesis, neuron maturation and migration, cofactor for &gt;200 enzymes</td>
</tr>
<tr>
<td>Selenium</td>
<td>Component of selenoproteins in brain, antioxidants</td>
</tr>
<tr>
<td>Iodine</td>
<td>Neuron differentiation and maturation, myelination, neurotransmission</td>
</tr>
<tr>
<td>Copper</td>
<td>Neurotransmission, brain energy metabolism, antioxidant</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Brain energy metabolism, myelination, neurotransmission</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
</tr>
<tr>
<td>LCPUFA (DHA, ARA)</td>
<td>Neurogenesis and growth, synaptogenesis, two major lipids of gray matter</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Major component of neuronal membrane, precursor for key second messengers</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>Myelination, major component of myelin sheath</td>
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<tr>
<td>Gangliosides</td>
<td>Component of neuronal membrane, signal transduction</td>
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<tr>
<td>B vitamins</td>
<td></td>
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<tr>
<td>Folate</td>
<td>Myelination, neural cell proliferation and differentiation, DNA biosynthesis</td>
</tr>
<tr>
<td>B12</td>
<td>Myelination, neural cell proliferation and differentiation, 1-carbon metabolism</td>
</tr>
<tr>
<td>Choline</td>
<td>Neurotransmitter synthesis, myelination, DNA methylation</td>
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<tr>
<td>Carotenoids</td>
<td></td>
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<tr>
<td>Lutein</td>
<td>Major carotenoid in brain, antioxidant</td>
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<tr>
<td>Proteins</td>
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<tr>
<td>Lactoferrin</td>
<td>Major iron-binding protein, major whey protein in human milk</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Breast milk protein important for immunity and emerging research suggests a role in myelination</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Emerging roles on brain likely via the microbiota-gut-brain connection</td>
</tr>
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</table>

**Iron**

Iron, as an essential structural component of the hemoglobin molecule, plays a critical role in transporting oxygen throughout the body. The role of iron as a vital nutrient in brain development is highlighted by its sensitive dose-response window; both iron excess and deficiency may induce abnormal brain development. Iron deficiency alters myelination (WM), monoamine neurotransmitter synthesis (striatal-frontal), and neuronal and glial energy metabolism (hippocampal-frontal) [17]. These brain processes are correlated with developmental behaviors, such as speed of processing (myelination), changes in motor and affect (monoamines), and recognition memory (hippocampus). Infant iron deficiency anemia is a risk factor for both short- and long-term cognitive impairment. These effects may persist even if treatment is provided during infancy, highlighting the gravity of the perinatal role of iron. In multiple longitudinal
studies, children with indication of chronic severe iron deficiency in infancy were found to have poorer outcomes than those without. Lack of exposure to iron perinatally resulted in long-term IQ effects, lower motor scores, more grade repetition, anxiety or depression, and social problems [18]. These outcomes persisted despite the introduction of iron therapy in response to anemia in infancy [18]. The mechanism of the perinatal role of iron has been demonstrated robustly in rodent models. Gestational and neonatal iron deficiency resulted in deficits in hippocampal dendritic branching, which persisted in adulthood despite restoring iron levels to baseline [19]. In addition, even minimal iron deficiency during prenatal and early postnatal development decreases myelin synthesis and alters myelin composition, which is also not corrected with iron therapy [16].

**Choline**
Choline is a micronutrient highly interrelated with folic acid, vitamin B₁₂, and methionine that is integral in spinal cord and brain development during the perinatal period. Specifically, choline is the precursor of the (1) neurotransmitter acetylcholine, (2) structural phospholipids phosphatidylcholine and sphingomyelin which act as precursors for intracellular messengers, and (3) two signaling lipids, sphingosylphosphocholine and platelet-activating factor. The fetus receives choline across the placenta prenatally and continues to receive high amounts of choline compounds via breast milk postnatally. Rodent studies demonstrate that choline supplementation in utero during the critical window supports hippocampal development (embryonic days 11–18), increases cell proliferation and decreases apoptosis in hippocampal progenitor cells. Rodent studies also show that exposure to extra choline in utero and perinatally leads to hippocampal structure changes that are protective against the cognitive and behavioral effects of prenatal stress and alcohol exposure [20], and even result in lifelong improvements in long-term potentiation and visuospatial auditory memory [21]. In a retrospective study, women in the lowest quartile of daily choline intake had a 4-fold greater risk of having a baby with a neural tube defect than the women in the highest quartile for intake [22]. While the mechanism through which choline contributes to permanent changes has not been fully elucidated, the likely mechanisms for the effects of choline come from the cascade effects of its role in DNA methylation, altered gene expression, and the downstream-associated change in stem cell proliferation and differentiation.

**Long-Chain Polyunsaturated Fatty Acids**
The LCPUFAs, such as docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6), are essential for brain development during both the prenatal time period as well as the first 2 years of life [23, 24]. In particular, DHA is
highly concentrated in the membrane lipids of the WM and gray matter (GM) of the brain and has been implicated as highly essential for WM myelination. In addition, since DHA is abundant in breast milk, it is not surprising that extensive studies have reported cognitive benefits of DHA, including improved working memory [23] and problem-solving skills [24] in infants receiving DHA-supplemented formula. In particular, numerous studies have evaluated the potential benefits of DHA on visual acuity. The DIAMOND (DHA Intake and Measurement of Neural Development) study, a double-masked randomized controlled clinical trial using a dose escalation design, evaluated the potential beneficial effects of DHA on visual acuity [25]. Improved visual acuity was observed only in the group with DHA supplementation of infant formula at 0.32% of total fatty acids. In contrast, Rosenfeld et al. [26] conducted a meta-analysis of 4 clinical trials focusing on DHA; no effects on Bayley Developmental scores at 18 months of age was observed. However, Hoffman et al. [27] suggested that the ratio of DHA and ARA supplement in formula may be of critical importance in determining the potential outcome of cognitive function. Liao et al. [28] utilized event-related potential (ERP) evaluating cognitive improvements in infants receiving control versus DHA-enriched formula at birth and cognitive assessments at 5.5 years of age. Although behavioral assessments (reaction time and accuracy) reveal no statistical differences among groups, ERP shows that the children receiving enriched formula at birth exhibited a greater P2 amplitude, suggesting better visual functioning when compared to the control group. They subsequently concluded that the limited sensitivity of behavioral assessments may contribute to the observed discrepancies between behavioral assessments and ERP and called for more sensitive approaches. To this end, imaging methods, such as MR (to be discussed below), may provide an alternative and sensitive means to assess brain functional development in the first years of life.

Noninvasive Imaging Approaches

While behavioral and cognitive assessments have been widely employed as the outcome measures for most studies focusing on discerning potential beneficial effects of nutrients on brain functional development, they suffer from several inherent limitations. First, a large sample size is typically needed given the anticipated large interrater and intersubject variability. Second, the behavioral repertoire of young infants is relatively limited, making it difficult to objectively assess higher-order brain functional development. Finally, these approaches do not provide a direct assessment of the neural substrates underlying brain functional maturation. Therefore, a sensitive and objective means to assess brain
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functional development is greatly needed to be able to rigorously, objectively, and quantitatively assess early brain development.

Magnetic resonance imaging (MRI) is a noninvasive imaging modality that is highly versatile and capable of providing detailed anatomical information with exquisite soft-tissue contrast. MR has no radiation and no known adverse effects, enabling longitudinal and noninvasive characterization of normal brain development. However, MR is highly sensitive to motion artifacts; slight motion could lead to poor image quality. While adult subjects can tolerate the immobility required for MRI, this requirement is clearly difficult for children, particularly for toddlers, to comply. Therefore, while MR is an ideal tool to reveal the underlying neural substrates of early brain development, it is only recently that MRI of typically developing children without sedation has become a reality. With dedicated staff and infrastructures, our team has developed strategies that enable imaging of typically developing children from birth to teens without sedation [29]. In particular, 2 main categories of MRI approaches have been employed to characterize structural and functional development during the first years of life. An overview of these imaging approaches, results on early brain structural and functional maturation during the first years of life, and how these approaches may aid to nutritional research focusing on early brain development will be provided below.

Structural Development during the First Years of Life

The total brain volume increases 101% in the first year, followed by a 15% increase in the second year [3]. The increase in GM volume (149%) accounts for the majority of the observed increased total body volume with only about 11% increases in WM during the first year [3]. Furthermore, ample evidence demonstrates links between MR morphological measures and functional maturation. Reduced cortical GM density during adolescence and early adulthood [30] is temporally correlated with postmortem findings of increased synaptic pruning. Significant correlations between GM volume in the frontal lobe and children’s performance on a verbal learning task have also been reported [31]. Similar observations with cortical thickness (CT) have also been made where cortical thinning occurs with age as part of normal brain maturation [32]. Thinning of frontal and parietal cortices is associated with improvements in performance on language tasks [33]. While these previous results underscore the importance of structural features of early brain development, detailed characterization of brain structural growth during the first years of life has been a daunting task, particularly using a longitudinal protocol. Figure 1 shows T1w (upper row) and T2w (lower row) images of a subject imaged starting from 2 weeks to 6 years of age, demonstrating the remarkable growth of our brain during the first 6 years of life. Table 2 provides quantitative measures of cortical GM volume, CT, and cortical
surface area (SA), where SA × CT = GM volume, from a cohort of typically developing children (2 weeks to 6 years of age) undergoing a longitudinal MRI study. Although both GM volume and CT have been widely used to reveal brain structural growth, relatively few results on SA are available. Therefore, the implications of brain SA expansion in relation to cognitive development are yet to be rigorously determined. In particular, it has been demonstrated that brain atrophy typically observed in elderly and demented patients is largely attributed by CT thinning with little change in brain SA [34]. In contrast, computational models implicate that brain SA expansion is more efficient to facilitate brain connectivity than increasing CT [35]. In the context of early brain development where synaptogenesis is one of the main developmental processes, one would hypothesize that not only marked brain SA expansion is present, but also its increase should outpace cortical thickening. Indeed, carefully examining the results shown in Table 2, several important features emerge. First, the total cortical GM volume
has almost doubled by the 9th month, followed by a much slower growth pace after the first year of life. By 5 years of age, only a negligible increase in GM volume is observed. Second, CT increases from 2.21 ± 0.14 mm at birth to 2.72 ± 0.18 mm at 1 year, suggesting cortical thickening during the first year of life. However, CT is reduced (cortical thinning) starting from the 18th month of life till about 3 years of age (2.62 ± 0.07 mm) and finally becomes more stable after 3 years of age. Third, although cortical thickening is observed during the first year of life, the increase in CT is relatively small (23%) compared to the increase in cortical GM volume (115%) at the same time period. In other words, the increased cortical GM during the first year of life should be largely attributed by brain SA expansion. Indeed, brain SA exhibits a similar growth trajectory as that of cortical GM volume during the first 6 years of life; a marked increase during the first several years of life, followed by a slower brain SA expansion. Together, our results suggest that brain SA expansion is the dominating factor contributing to an increased cortical GM and underscore the potential biological significance of SA expansion during early brain development. Nevertheless, more studies will be needed to determine the potential cognitive implications of SA expansion.

Although the above discussion on brain structural development only focused on 3 structural parameters, it is worth pointing out that additional anatomical features, including but not limited to cortical folding [36], gyrification [37], and sulcal depth [38], can also be obtained using MRI (Fig. 2), demonstrating the capability of MRI in characterizing early brain development.

Myelination is a critical maturation step to ensure rapid and efficient information communication through WM. Successful connection among brain cortical regions likely plays a critical role for the maturation of brain functions. Using diffusion tensor imaging (DTI), an MRI approach, numerous reports have revealed temporal processes of WM myelination [5, 39, 40]. During the early postnatal period, WM first exhibits significantly lower fractional anisotropy (FA), a quantitative measure of directional water mobility, followed by an increased FA with age [5, 39]. The central WM consistently exhibits a higher FA than the peripheral WM in neonates [39]. Huang et al. [41] reported that limbic fibers developed first while the association fibers developed last and commissural and projection fiber tracts are forming from anterior to posterior regions of the brain. Significant associations were also found for WM tracts that connect brain regions known to support working memory in older children and adults (genu, anterior cingulum, and arcuate fasciculus). Better working memory was associated with higher FA and lower radial diffusivity values in these WM tracts [13].

Despite its wide applicability to characterize WM maturation during early brain development, the spatial resolution of DTI is generally lower (1.5 mm³) than
that of anatomical images (0.8 mm³). As a result, its ability to characterize myelin content in cortical GM is substantially limited. Yet, quantitative measures of cortical myelin content have been implicated to be associated with brain cognition; a higher cortical myelin content represents a matured cortex and vice versa. To mitigate this difficulty, Glasser and Van Essen have recently proposed a new approach capable of revealing regional cortical myelin content [42]. Although more studies are needed to identify the links between cortical myelin content and cognition, as shown in Table 1, several nutrients (iron, DHA, choline, and sphingomyelin, for example) have been implicated to be of importance for myelination processes. Therefore, in addition to DTI, measures of myelin contents should be included for the assessments of nutritional impacts on brain cognitive development.

**Functional Brain Development during the First Years of Life**

While it is critically important to determine how different nutrients may impact on structural maturation, equally important is to discern the interplay between cognitive maturation and nutrients during early infancy. Although a number of
behavioral and cognitive batteries are currently available to assess brain functional development, as outlined above, these batteries may not be applicable during early infancy. Alternatively, functional MRI (fMRI) or, more specifically, resting state fMRI (rsfMRI) is a perfect tool to characterize brain functional maturation processes in children. In particular, unlike conventional fMRI where a subject’s cooperation to perform predefined cognitive tasks is needed, no specific requirements other than to keep still are needed for rsfMRI. Using rsfMRI, various brain functional networks, including both basic (motor, sensory, visual, and auditory) and higher-order (default mode, attention, executive control, and salience) networks, have been consistently observed in adults. More importantly, other and our teams have employed rsfMRI to examine the development of resting state connectivity in early infancy [9, 43–45]. In a whole-brain topological analysis of 147 healthy subjects from 3 weeks to 2 years of age, we observed small-world topology immediately after birth, with continued maturation during the first 2 years of life [44]. The brain functional topology appears to be more regionally based, without evidence of substantial, long-distance connections before 1 year of age; connectivity becomes more evenly distributed, with increased long-distance connections in year 2 [44]. Furthermore, we reported [46] temporal maturation of major brain functional networks observed in adults during the first year of life, including the sensorimotor (SM), auditory (AN), visual (V1, V2, V3), default mode (DMN), salience (SA), and frontoparietal frontal control (FPC) networks. Based on the growth rates, these networks were classified into 4 different groups. Group 1 includes the AN and SM networks, which are the slowest-growing networks, while group 2 includes V1 and V2, representing the fastest-growing networks. Group 3 includes V3 and DMN, and group 4 includes SA and FPC, reflecting networks with moderate growth rates. Although groups 1 and 4 include networks with slower growth rates, they are distinctly different in functions. It is most likely that group 1 represents the almost matured networks while group 4 reflects the networks that are yet to be developed.

Given the critical role of language function on the development of multiple higher-order functional domains, insights into language maturation during the first years of life could be of critical importance. Homologous language regions (Broca’s and Wernicke’s areas) between the two hemispheres have been reported to be functionally connected (functional symmetry) immediately after birth [47, 48]. However, this pattern shifted toward a strong connection between Broca’s and Wernicke’s areas within the same hemisphere but not across the two hemispheres (functional asymmetry), likely reflecting language lateralization [49]. Using a longitudinal design, we evaluated how functional connectivity in the language areas progresses from a symmetric connection at birth to an asymmetric connection between the two hemispheres during early brain develop-
ment. Both Broca’s and Wernicke’s areas show an increased symmetrical connection in the first year with a peak at approximately 11.5 months of age, followed by a decrease in the second year. Furthermore, there was a significant correlation between individual’s time to peak symmetry between these two regions ($p = 0.011$). These findings reveal the complexity and nonlinearity of brain functional developments. More importantly, our results suggest that language lateralization, a critical maturation milestone of language functional development, may be present by the end of the first year of life.

While the aforementioned studies have largely focused on characterizing early brain functional maturation processes without considering factors such as nutrients, these studies demonstrate that rsfMRI can serve as an indispensable tool evaluating how different nutrients may alter functional maturation trajectories during early brain development. Specifically, by collecting breast milk samples, dietary intake, and information on feeding practices concurrently with MR images and detailed behavioral/cognitive assessments, the interaction of these important parameters can potentially be discerned.

**Gut-Brain Axis**

Recent discovery of bidirectional biochemical signaling between the gastrointestinal tract (including gut flora) and the central nervous system has attracted great interdisciplinary efforts to uncover the gut-brain axis (GBA). While research has primarily been conducted in animals thus far, insights have revealed a highly complex communication system that not only maintains gastrointestinal homeostasis [50] but also links peripheral intestine functions to affect motivation and higher cognitive functions [51, 52]. In the absence of microbiota, mice have either an elevated or suppressed stress response level, correlated with abnormal corticosterone concentrations, depending on the wild-type microbiome of the mouse strain [53, 54]. This defect can be returned to baseline with colonization with a specific bacterial species (*Bifidobacterium infantis*) but not with monocolonization with *Escherichia coli*, demonstrating that signals from specific bacteria critically impact GBA [54]. In human studies, a 4-week probiotic consumption intervention decreased emotional stimulation in an emotional faces fMRI task, specifically affecting the primary interoceptive and somatosensory regions of the brain [55]. Most relevant to understanding the relationship between nutrition and brain development is a recent study that investigated the relationship between gut colonization and brain development for children 1 and 2 years of age. Combining microbiome analysis, MR images, and cognitive testing, human microbial composition at year 1 predicts cognitive
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performance at year 2 [56]. Further work in understanding GBA is critical in finding out how the axis breaks down in disorders that are already linked to abnormal GI functioning, such as autism, depression, and anxiety [52], as well as understanding the mechanisms for the role of nutrition in brain development and cognition.

Conclusions

The ability to rigorously characterize both structural and functional maturation during the first years of life using MRI is likely to have profound implications on how factors such as nutrition could enhance healthy growth of our brain. The Baby Connectome Project (BCP) – Enriched study, a study specifically designed to discover the complex interaction of nutrition, GBA and early brain maturation, should offer invaluable insights into the interaction of these essential physiological parameters. This knowledge will potentially guide our ability to supplement critical nutrients in early childhood, optimizing both timing and dosage, to promote the healthy growth of our brain during the first years of life.

Disclosure Statement


References


Abstract
Gut immune function conditions the development of local and systemic diseases that result from defects in immune regulation, such as inflammatory bowel disease, allergy and obesity. As epidemiological studies support the developmental origin of health and disease, deciphering the critical factors modulating gut immune development should allow the advance of primary prevention strategies specifically adapted to the early-life immune system. Here, we will review gut mucosal immunity development and cover in more detail the recent understanding of the impact of early nutrition on this process. We will emphasize how nutrition can shape microbiota composition and metabolic function and thereby the production of metabolites with immune-modulatory properties. We will also focus on the role of dietary compounds recently demonstrated to be essential in immune development and function, such as dietary antigens, vitamin A, and aryl hydrocarbon receptor ligands. Finally, we will discuss that early-life physiologic food for mammals contains factors capable of compensating for neonatal immune deficiencies, but also factors that are decisive for immune maturation towards a maternal milk-independent and efficient immune system.

Introduction
Early-life gut immune function can be qualified as deficient according to the high susceptibility of neonates and infants to enteric infections. The high incidence of food allergy in infants further highlights a deficient capacity to mount
oral tolerance. Mucosal immunity will mature in postnatal life to evolve, ideally, to mount regulatory immune responses towards dietary and nonpathogenic microbial antigens and inflammatory responses against pathogens. In parallel to immune system maturation, the gut develops from a nearly sterile environment at birth to harboring a microbiota that is very simple and highly variable and eventually reaches a dense, complex, and stable microbiota around 3 years of life [1]. Both epidemiological and experimental studies have linked perturbations in gut microbiota composition in early life with the risk of developing immune-mediated disease in later life [reviewed in 2]. Infants at risk of asthma show a reduction in the relative abundance of certain bacterial genera during their first 3 months of life, while no major difference in microbiota composition was found at older ages. Clinical studies also suggest that disturbances in the intestinal flora early in life, caused by cesarean section delivery or early antibiotic exposure, may contribute to the development of diseases such as food allergy, inflammatory bowel disease, and types 1 and 2 diabetes [2].

Here, we will review experimental evidence that nutrition can affect gut immune ontogeny directly and indirectly by shaping microbiota composition, with lifelong impact on immune homeostasis.

**Gut Immune Ontogeny**

The gut epithelium progresses from being flat and poorly proliferating towards a highly proliferative epithelium, with crypt and villus architecture dramatically expanding the absorptive surface. This development is completed at birth in humans while still developing postnatally in mice [reviewed in 3]. A high gut permeability is found in the neonate, both in mice and humans. In mice, few goblet and Paneth cells are present at birth and there is a low secretion of IgA in the gut lumen. Neonatal enterocytes however produce CRAMP (cathelicidin-related antimicrobial peptide) that acts as an antimicrobial defense mechanism specific to the early-life period, as it is also found in breast milk and the vernix caseosa. The high susceptibility of preterm neonates to necrotizing enterocolitis shows that an immature gut epithelium is susceptible to highly inflammatory responses; however, regulatory mechanisms such as decreased Toll-like receptor (TLR), TLR3 and TLR4, expression and signaling are found in neonates born at term, which contribute to dampening inflammatory responses upon microbial colonization [3].

Peyer’s patches and mesenteric lymph nodes (MLNs) develop in utero while isolated lymphoid follicles (ILF) do so after birth [4]. A common scheme operates for lymphoid tissue formation: a stromal cell produces chemokines
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(CXCL13) which will attract CXCR5-expressing lymphoid tissue inducer (LTi) cells of hematopoietic origin (more recently known as a subset of innate lymphoid cells [ILC], ILC3, expressing RAR-related orphan receptor γ [RORγ]t). Binding of lymphotxin (LT) αβ secreted by LTi to LTβ receptors expressed by stromal cells induces chemokine secretion and adhesion molecule expression needed for the attraction and retention of additional hematopoietic cells, leading to lymph node growth [4]. Peyer’s patches require CD11c+ cells expressing the receptor tyrosine kinase RET, in addition to LTi and stromal cells, for their formation. ILF originate from cryptopatches, which are a cluster of LTi in the lamina propria. These expand after birth in the presence of B cells and a few T cells to become ILF [4].

A comprehensive analysis of the emergence of immune cells in murine neonatal small intestine was recently undertaken [reviewed in 3]. This showed that myeloid cells are found in the lamina propria at birth, and their numbers remain stable postnatally. We further found that, although in similar frequency in neonatal MLNs as compared to the adult, the capacity of neonatal CD103+ dendritic cells (DCs) to metabolize retinol was significantly impaired [5]. Importantly, we found this defect responsible for inefficient oral tolerance induction in the neonate [5]. After a massive recruitment of CD4+ T cells and B lymphocytes into the gut mucosa during the first 2 days of life, B cells were found to continue to expand, while TCR-αβ lymphocytes did so only after weaning. Torrow and Hornef [3] also found that, before weaning, CD4+ T lymphocytes were mostly found in Peyer’s patches, leaving the lamina propria relatively empty compared to the adult. They further uncovered that these lymphocytes exhibit a naïve phenotype until weaning, due to active suppression by FoxP3+ regulatory T cells (Tregs) and maternal milk IgA. Similar to mice, a recent analysis of T-cell representation and differentiation in tissues of infants from 2 months to 2 years of age revealed a higher proportion of naïve and regulatory T cells in all tissues compared to the adult [6]. Except for the subset of ILC3 involved in lymphoid tissue morphogenesis, LTi, limited data are currently available addressing the representation and function of ILCs in neonatal gut mucosa.

In summary, early postnatal life gut mucosal immunity is characterized by a leaky barrier with poorly developed innate and adaptative effector mechanisms, which are kept under control by regulatory cells. Breastfeeding is therefore critical for the prevention of infectious diseases in young infants as it provides the breastfed infant with antigen-specific (IgA and IgG) and nonantigen-specific antimicrobial molecules (lysozyme, lactoferrin, oligosaccharides, and leukocytes) [7]. Despite the high proportion of Tregs in the neonatal gut mucosa, oral tolerance can hardly be induced in neonates [reviewed in 7], and highly inflammatory reactions such as necrotizing enterocolitis can take place in the neonatal gut. Here
also, breast milk compensates for the lack of molecules involved in immunoregulation in the neonatal gut. TGF-β, epidermal growth factor, human milk oligosaccharides (HMOs), and maternal IgG and IgA present in breast milk were indeed found to exert anti-inflammatory and immunoregulatory effects [7, 8]. In the next parts of this review, we will analyze how nutrition is involved in neonatal gut immune maturation towards an autonomous system, no longer dependent on breast milk to mount efficient regulatory and effector immune responses.

Impact of Nutrition on Gut Mucosal Immune Ontogeny through Microbiota Shaping

Gut Microbiota Ontogeny
Besides the mode of delivery, known to strongly affect colonization of the neonatal intestine within the first days after birth, nutrition is the key factor directing the early microbiota composition and function [reviewed in 1]. The gut flora in breastfed infants is usually dominated by *Bifidobacterium* and *Lactobacillus* species, while formula-fed infants harbor a more diverse gut microbiota with increased abundance of *Escherichia coli*, *Clostridium*, and *Bacteroides*. The microbiomes of newborns and young infants are enriched in genes required for the degradation of sugars from breast milk, such as HMOs. Upon weaning, the microbiota functionally maturates by a decrease in the relative abundance of genes involved in the degradation of HMO and enrichment of genes involved in the degradation of complex sugars and starch. Cessation of breastfeeding, not solid food introduction, is critical for this shift.

Mechanisms of Microbiota–Driven Immune Shaping
Germ-free (GF) mice represent an extreme situation that illustrates the necessity of microbiota for gut immune development. Gut microbiota was shown to be necessary for ILF formation in the small intestine and the development of MLN and Peyer’s patches. Differentiation of immune responses is also dependent on microbial colonization. Microbiota is necessary for IgA secretion, regulation of IgE responses, differentiation of Th17 and Th1 cells, and RORγt+ Treg expansion in the colon [9]. Gut microbial composition exerts direct effects on the immune system through microorganism-associated molecular pattern (MAMP) signaling and indirectly via the production of metabolites. An example of a well-studied MAMP is polysaccharide A from *Bacteroides fragilis* that was found to promote Treg differentiation and MYD88-signaling involved in epithelium repair and antimicrobial peptide secretion [9]. Fermentation of dietary fibers in the colon by anaerobic bacteria, such as clostridia and bifidobacteria,
generate short-chain fatty acids (SCFAs) including butyrate, acetate, and propionate. SCFAs signal through G-protein-coupled receptors, such as GPR43, GPR41, and GPR109A, present on epithelial and immune cells, and via inhibition of histone deacetylases, with long-term consequences through epigenetic modification. Among many of their reported immune effects, SCFAs promote colonic Treg differentiation, mucus production, and IgA secretion (Fig. 1a). Commensals, particularly Lactobacillus, metabolize tryptophan, an essential amino acid that is a common constituent of protein-based foods such as eggs, fish, meat, and cheese (Fig. 1b). The metabolites, which are indole derivatives, bind aryl hydrocarbon receptor (AhR). AhR was found to be necessary for ILC3 function both in postnatal development of ILF and IL-22 secretion necessary for gut barrier function and protection from Citrobacter infection and colitis [9].

While most of the studies addressing the role of the gut microbiota on immune maturation have been performed in adults and observations extrapolated to the developing neonate, some recent publications have specifically addressed the impact of colonization in early life on immune ontogeny [reviewed in 2]. These have

**Fig. 1.** Impact of food on immune ontogeny. **a** Short-chain fatty acids (SCFAs) stimulate regulatory T-cell expansion, IgA, and mucus secretion, gut epithelium barrier function, antimicrobial peptide secretion (AMP), and innate lymphoid cell (ILC)3 function and induce resistance to food allergy and gut inflammatory disease. Human milk oligosaccharides (HMO) are present in human milk and stimulate the growth of bifidobacteria that can metabolize HMO into SCFA. After weaning, the metabolic function of bifidobacteria changes, and they become capable of metabolizing complex sugars from dietary fibers, similarly to clostridia found in microbiota of older children. **b** Aryl hydrocarbon receptor (AhR) ligands bind to AhR receptor expressed on ILC3. They stimulate the postnatal formation of isolated lymphoid follicles (ILF) and IL-22 secretion necessary for gut barrier function and protection from Citrobacter infection and colitis. AhR ligands are found in cruciferous vegetables such as broccoli and cabbage. They can also be produced by some commensals, such as Lactobacillus, which metabolize tryptophan from protein-based foods into indole derivatives. In breastfed infants, AhR ligands can originate from maternal microbiota metabolites with maternal milk immunoglobulin helping in the transfer of these metabolites to the neonate. Breast milk may also contributes to AhR-mediated immune ontogeny by stimulating the growth of Lactobacillus. **c** After weaning, antigens derived from solid food are necessary to populate the small intestine with induced Tregs. Tregs specific to dietary antigens can be induced by oral exposure. Before weaning, oral tolerance can be induced to antigens from the maternal diet which are present in breast milk. This requires the presence of additional cofactors in breast milk such as TGF-β, vitamin A, and IgG. Vitamin A increases gut barrier function, the capacity of dendritic cells (DCs) to metabolize vitamin A into retinoic acid and Th1 differentiation. Antigens bound to IgG are better transported across the epithelium and induce FoxP3 Tregs that are responsible for potent and long-lasting tolerance. After weaning, Treg induction towards oral antigen is favored by SCFAs. These induce TGF-β secretion from epithelium and stimulate retinoic acid formation from vitamin A by DCs.

*(For figure 1 see next page.)*
started to show clear specificities of early-life immune responses to microbiota colonization and highlight the concept of a window of opportunity to induce a lifelong effect on immune homeostasis by shaping the microbiota in early life. Thus, GF mice conventionalized during adult life exhibit a different transcriptional profile in jejunum and colon compared to conventionally raised mice. High IgE found in GF mice can only be normalized if colonization occurs before 4 weeks.
of age. Another detailed mechanistic study showed that colonization of GF mice with gut microbiota in the first 2 weeks of life, but not in adults, was sufficient to protect mice from increased susceptibility to colitis due to mucosal invariant natural killer T (iNKT) cell accumulation. The protective effect of colonization in early life could also be induced by *B. fragilis* monocolonization and depends on polysaccharide A signaling. Colonization with *Clostridium* during weaning induces colonic Treg and fecal IgA as well as IL-22 production by RORγt+ ILCs and T lymphocytes, promoting gut barrier function and resistance to food allergy [10]. In contrast, neonatal colonization with specific strains of the commensal *E. coli* impairs oral tolerance induction by affecting intestinal permeability and the balance of tolerogenic DCs and Tregs through the production of a genotoxin [11].

Age-specific mechanisms of action of microbiota-driven immune shaping were also found in neonates. Gomez de Aguero et al. [12] found that maternal microbiota generates AhR-binding metabolites that are transferred in utero and postnatally through breast milk and induce mononuclear cells and ILC3 expansion. The latter effect was found to be increased by maternal antibodies in the milk. The strong impact of gut microbiota on immune function has stimulated research on the potential to promote infant microbiota development by oral administration of probiotics to induce health benefits. Despite an increase in probiotic administration, data supporting their efficacy is lacking [1]. Another strategy is to promote the growth of beneficial bacteria using prebiotics.

*Early Nutrition Driving Microbiota Composition and Function*

Breast milk contains $10^2$ to $10^4$ viable bacteria/mL that will directly affect the establishment of the neonatal microbiota. It also contains prebiotic and immunologic compounds that can alter colonization patterns in the neonate. In particular, HMOs stimulate the growth of bifidobacteria, which metabolize these oligosaccharides into SCFAs that favor immune regulation (Fig. 1a). HMO also have the capacity to modify the gene expression involved in metabolic function in commensals and thereby their secretion of metabolites which can affect growth [13] and inflammation [14]. Metabolized HMOs are also beneficial for other commensals that do not directly degrade HMOs [13]. In this context, dietary and synthetic oligosaccharides are the object of studies to assess similar early-life immune regulation, with promising results [1]. Breast milk secretory IgA antibodies are specific for an array of common intestinal pathogens and commensals due to the selective migration of B cells originating from the mucosal membranes to the mammary gland. In addition to providing excellent passive mucosal immunity to neonates, maternal IgA and other molecules found in breast milk with antimicrobial properties, such as lactoferrin and lysozyme, will shape the microbiota composition of the breastfed infant [7].
Direct Impact of Nutrition on Gut Mucosal Immune Ontogeny

Here, we will focus on the impact of breastmilk and selected nutrients that have recently been the focus of experimental studies in early life for their impact on immune ontogeny, that is, dietary antigens, vitamin A, RORγt, and AhR ligands.

Solid Food and Dietary Antigens

The role of dietary antigens on gut mucosal immunity ontogeny has been assessed by comparing mice that were free of specific pathogens, that were devoid of microbiota (GF mice), or that were devoid of both microbiota and dietary-derived antigen (antigen-free mice) from birth [15]. Others also studied the impact of dietary antigen after weaning on immune ontogeny of specific pathogen-free mice [16]. These studies demonstrate that, after weaning, dietary-derived antigens are necessary and sufficient to stimulate memory CD4+ T lymphocytes and peripherally induced Tregs to populate small-intestine lamina propria. They are required for controlling the Th2 immune response and susceptibility to food allergy and necessary for IgA and IgG secretion. Evidence that exposure to diet-derived proteins is important to induce immune regulation also arises from intervention studies analyzing the impact of food diversification in the first year of life [17]. These observations, made both in mice and in humans, highlight that one should consider the administration of an extensively hydrolyzed formula to infants with allergies with care, as this nutritional approach may decrease regulatory function of the gut.

The shaping of immune reactivity by induction of oral tolerance to specific antigens during the period of immune ontogeny, was recently reviewed [17]. Egg introduction between 4 and 6 months was able to prevent from egg allergy, and peanut introduction between 4 and 11 months was able to prevent from peanut allergy [17]. However, the results obtained for the prevention of peanut allergy required adherence to the protocol that would hardly be achievable in the daily life [17]. Furthermore, the early introduction of other allergens such as fish or milk did not prevent allergy, and early introduction of gluten did not reduce the risk of celiac disease [17]. Overall, these data show that promoting tolerance by oral antigen exposure during immune ontogeny is possible, but that additional cofactors are required to enhance the chance of success. In this regard, the role of breast milk factors in promoting oral tolerance has attracted increased interest. Antigens from the maternal diet are found in breast milk at concentrations 1,000-fold lower (ng/mL) than antigen levels in formula milk (mg/mL). Unexpectedly, we also found antigens of respiratory sources such as the house dust mite Dermatophagoides pteronyssinus (Der p) and Blomia tropicalis in breast milk, in similar amounts as dietary antigen [18, 19]. Since we detected Der p 1 in digestive fluid of healthy
adults [20], we propose that respiratory allergens are ingested by being trapped in the oropharynx or pulled back by the mucociliary epithelium and follow the same route as dietary antigens to the mammary gland. We have specifically addressed the factors in breast milk that could improve the chance of oral tolerance induction to dietary and respiratory antigens in rodents. We found that mice exposed to a few nanograms of egg ovalbumin (OVA) antigen through breast milk were protected from OVA-induced allergic airway disease and food allergy [21, 22]. Importantly, TGF-β from breast milk was necessary for oral tolerance induction [22]. We demonstrated protection to be more profound when OVA was transferred through the breast milk of OVA-immunized mothers than OVA-exposed nonimmunized mothers [23]. OVA-specific IgG in milk was necessary for protection during transfer of OVA though the gut barrier and the induction of a prolonged protection mediated by FoxP3 Tregs. We also identified a key role of vitamin A in breast milk in the process of neonatal gut immunity maturation (see below) [5]. Our more recent data further indicated that the nature of antigen found in breast milk could dramatically affect immune outcome. In contrast to the observation with OVA, the transfer of Der p 1 through breast milk induced Th2 immune response priming and increased susceptibility to allergic disease in adult mice [24]. Importantly, in a human birth cohort, the risk of allergic sensitization and respiratory allergies in children breastfed by mothers increased with Der p 1 levels in breast milk [18]. This observation stresses that not all the antigens in breast milk induce oral tolerance, and that there is a need to identify how maternal milk factors could be modulated to counteract the deleterious actions of some allergens. Ongoing intervention studies will help to decipher whether antigens in breast milk impact on immune tolerance induction [25].

**Vitamin A**

Vitamin A is found in animal-derived food such as milk, liver, and egg yolk, while its precursors, the carotenoids, are found in vegetables such as carrots and broccoli. Major advances have recently been made on the impact of vitamin A and its metabolite retinoic acid (RA) on immune homeostasis [7]. Specifically, RA promotes CD4+ T cell differentiation, supports the generation of IgA-secreting B cells, and mediates the balance between ILC3 and ILC2. RA also imprints gut-homing specificity on T and B cells to the small intestine. Vitamin A in conjunction with SCFA derived from fibers metabolized by microbiota, promoted oral tolerance and prevented food allergy [26]. We recently identified that neonatal mice are physiologically deficient in retinol; serum retinol levels then progressively increase and reach adult levels at 3 weeks due to breast milk vitamin A [5]. Low vitamin A levels at birth were found to be responsible for a leaky gut barrier, deficient RALDH expression by MLN CD103+ neonatal DCs, resulting in ineffi-
icient T-cell activation and the incapacity to induce oral tolerance in neonates [5]. Importantly, vitamin A supplementation was sufficient to accelerate gut epithelium differentiation in terms of architecture and barrier function while preserving the capacity of epithelial cells to digest milk sugars [5]. It also promoted immune maturation and allowed tolerance induction from birth, as observed in 3-week-old mice. Our observations also showed that vitamin A was involved in the maturation of the neonate’s immune responses towards Th1 immunity; this adds a dietary factor to the genetically programmed and microbiota-driven neonatal Th1 immune maturation [5]. Relevance of these data for the human is supported by reports on low retinol levels in healthy infants from well-nourished countries [5] and observational studies linking low retinol levels at birth with increased atopic risk in young adults [27]. In early postnatal life, vitamin A may also be involved in immune ontogeny by acting on lymphoid tissue organogenesis and development. Indeed, RA was shown to be necessary both for CXCL13 secretion by stromal cells [4], the first step in lymphoid tissue organogenesis, and for LTi differentiation and lymph node development [28]. This points to an important role of maternal intake of vitamin A and precursors during pregnancy in immune ontogeny and possibly in early postnatal life for lymph node development.

**Aryl Hydrocarbon Receptor and RAR-Related Orphan Receptor-γt Ligands**

RORγt is a master transcription factor for the development of lymphoid organs, Th17, and ILC3. Their presence in GF animals suggests that the microbiota is not a critical source of RORγt ligand [29]. The natural ligand was recently identified as a derivative of cholesterol, indicating that sterol metabolism may be essential for proper lymphoid tissue development in utero and possibly in early postnatal life for ILF.

In addition to binding microbiota-derived metabolites of tryptophan and pollutants, AhR binds dietary ligands contained in cruciferous vegetables, such as broccoli, that may then impact on gut immunity development after weaning. During the lactation period, AhR ligands in breast milk could originate from the maternal diet as well as from maternal microbiota-derived metabolites [12].

**Breast Milk**

Before weaning, breast milk supplies the neonate with antimicrobial and regulatory factors that complement its developing immune system. Breast milk is also providing the neonate with the factors necessary for immune maturation that the neonatal mammal would otherwise miss due to the lack of microbiota and solid-food-derived molecules. The maturating impact of breast milk was highlighted in a recent analysis of exfoliated gut epithelial cells in stools of 3-month-old children that were breastfed versus those formula fed and that showed a to-
tal of 1,214 genes differentially expressed between breastfed and formula-fed children [30]. Analysis of gene networks reflected broad differences with respect to signal transduction, cytoskeletal remodeling, cell adhesion, and immune response. Gut trophic factors such as epidermal growth factor, HMOs, and vitamin A found in human milk are most probably involved in these effects [7]. Breast milk exposes the infant to a variety of food antigens, and it contains ligands that are critical for lymphoid tissue development and immune function such as AhR ligands and vitamin A. It provides HMOs, as surrogates to fibers found in solid food, for commensals to produce SCFAs. Breast milk also delivers microbiota and food for commensal growth in the sterile neonate gut.

Conclusion

While the impact of gut microbiota-derived antigens and metabolites on gut mucosal immunity has largely been demonstrated, there is growing experimental and clinical evidence that diet may be as important for immune ontogeny and function. Before weaning, maternal milk, the physiological food for mammals, will reassemble all the varied exogenous factors required for immune maturation. Solid-food-derived antigens, vitamins, lipids, as well as food metabolites produced by the microbiota will then continue to shape the immune system function and dictate long-term susceptibility to local and systemic immune-mediated disease.

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Abstract
Recent demonstrations link clinical conditions, phenotypes alternating from inflammatory bowel disease, obesity, and allergic diseases to neurodevelopmental disorders, to aberrant gut microbiota composition. This has led to a growing interest in host-microbe crosstalk, characterizing the healthy microbiome and modifying its deviations at an early age. The rationale arises from the recognition of the intimate interrelationship between diet, immune system, and microbiome and the origins of human diseases. Before satisfactory preventive measures can be put in practice, important questions remain to be solved. First, we need more profound understanding of the complex mechanisms underlying these heterogeneous manifestations of immune-mediated and microbiome-associated chronic conditions. Second, long-term follow-up studies are required to determine whether the changes in the microbiome underlie the pathogenesis of noncommunicable diseases or are merely end results thereof, confronting the question of causality. This uncertainty notwithstanding, the complex and bidirectional interrelationship of the diet and the gut microbiota is becoming evident. Early exposures by the enteral route induce dynamic adaptive modifications in the microbiota composition and activity, which may carry long-term clinical impacts. Microbiota changes, again, control energy acquisition and storage and may contribute to gut immunological milieu; high-energy Western diets alter the microenvironment of the gut leading to propagation of the inflammatory tone and perturbation of gut barrier function and thereby to systemic low-grade inflammation. On this basis, rigorous clinical intervention studies, providing the ultimate answers to these questions, need accurate characterization of the immediate environment of the child, in
particular the early nutrition. The model of early nutrition for future studies is the healthy breastfed infant that remains healthy in the long term. Scientific interest is currently extending from the duration of breastfeeding to the composition of breast milk, which shows marked variation according to the mother’s immunological and metabolic health, antibiotic use, and mode of delivery. Human milk, rich in bioactive compounds, including health-promoting microbes and their optimal growth factors, human milk oligosaccharides, continues to afford tools to study diet-microbiota interactions for research aiming at reducing the risk of noncommunicable diseases.

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The Gut Barrier and the Healthy Microbiota

The mucosal surface of the gastrointestinal tract forms an important organ of host defense. In addition to its main physiological function, digestion and absorption of nutrients to meet the metabolic requirements and the demands of normal growth and development, the intestinal mucosa provides a protective interface between the internal environment and the constant challenge from antigens such as food and microorganisms from the outside environment. The maturation of balanced immunophysiological regulation here, however, depends on these external stimuli, particularly on the initial establishment of the gut microbiota. In point of fact, microbe contact in the perinatal period represents the most massive antigen exposure educating the physiological adaptation processes to the awaited postnatal environment.

One theory of the emergence of noncommunicable diseases involves disintegration in the maturation of the host key regulatory systems vis-à-vis the gut colonization process. Indeed, the microbiome is sensitive to environmental exposures displaying rapid adaptive competence, unlike the host genome [1]. The adaptations to an altered environment involve dynamic modifications in the microbiota composition and activity.

On this basis, the definition the healthy versus aberrant gut microbiota composition is unfeasible without considering the immediate environment of the child. Indeed, fundamental determinants of the infant gut colonization include maternal health and nutrition, the mode of delivery, early feeding, and antibiotic use.

Gut Microbiota: A Target for Preventive and Therapeutic Measures?

The industrialized societies worldwide are facing epidemics of diet-related chronic diseases, noncommunicable diseases, such as allergic, autoimmune, and inflammatory diseases. These conditions have been inextricably associated with
an aberrant compositional development of gut microbiota, dysbiosis. Furthermore, early-life exposures that are known to perturb gut colonization, including cesarean section delivery and antibiotic use, have been consistently linked to increased risk of noncommunicable disease [2, 3]. Especially during critical stages of development, dysbiosis induces lasting alterations in the immune and metabolic phenotype as well as neural pathways [4, 5], example manifestations including obesity, type 1 diabetes, asthma, allergies, and even neurodevelopmental disorders [6].

However, our knowledge of the cascade of events underlying the pathophysiology of these conditions with different target organs, onset age, and prognosis is by no means satisfactory. We need more profound understanding of the complex mechanisms underlying these heterogeneous manifestations of immune-mediated and microbiome-associated chronic conditions. Importantly, the question of causality needs to be given top priority in future research activities [7].

Thus, the microbiota forms a moving target for any preventive and therapeutic measures, as we do not know whether the changes in the microbiome underlie the pathogenesis of noncommunicable diseases or are merely a result thereof. Indisputably, the proof of causality requires clinical intervention studies in humans in different populations with rigorous and detailed documentation of the environment the infant is exposed to, the major determinant being early nutrition.

**Infant Gut Microbiota: Origin and Determinants of the Composition**

While the colonization process in the infant gut has been intensively studied, the early events guiding the microbiome development have only recently become uncovered. A stepwise process can characterize the establishment of the gut microbiota (Fig. 1). The initial inoculum before, at, and following birth involves mainly facultative anaerobic bacteria. Indeed, recent advances indicate that microbial colonization of the gut may start already during the fetal period. The first colonizing microbes present in amniotic fluid can also be recovered in meconium and belong to the *Escherichia* genus and lactic acid bacteria, including members of the genera *Leuconostoc, Enterococcus,* and *Lactococcus* [8].

Thereafter, the exposure to specific species in neonates is facilitated by the mode of delivery: vaginally delivered newborns harbor microbes from the vagina including *Prevotella* and *Lactobacillus* and also the genera *Bacteroides, Bifidobacterium, Parabacteroides,* and *Escherichia*. The maternal gut also appears to be an important source of early colonizing bacteria: 72% of gut bacteria in
vaginally delivered newborns are of maternal intestinal origin versus 41% in subjects born by cesarean section [9]. Indeed, newborns delivered by cesarean section are frequently colonized by bacteria associated with the maternal skin and mouth or the environment [9, 10]. Cohort studies from Europe and North America document reduced fecal abundance of *Bacteroides* or reduced diversity of Bacteroidetes phylum in infant gut following cesarean section delivery [11, 12]. In these, the fecal microbiome contains among others *Enterobacter, Staphylococcus*, including *S. aureus*, *Streptococcus*, and *Veillonella*, while *Bifidobacterium* are less abundant in cesarean-born than vaginally born infants [11, 12]. Cesarean-born infants also harbor more *Clostridium difficile*, and this may be mediating the dysbiosis frequently detected in these [12].

This initial colonization process directs the later microbiota succession and health of the infant [13]. Later in infancy, *Bacteroides, Bifidobacterium, Parabacteroides*, and *Escherichia/Shigella* species are abundant [9]. Maternal intrapartum antibiotic therapy or prophylaxis has also been reported to significantly perturb early gut colonization patterns and result in reduced diversity and low abundance of Actinobacteria among early gut microbiota [14, 15]. The detri-

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**Fig. 1.** The progression of gut colonization and the child’s risk of developing noncommunicable diseases. Key risk factors during the perinatal period and infancy include unfavorable nutritional environment during pregnancy, being born preterm or by cesarean section, or being devoid of important immunomodulatory compounds of breast milk. Resilience to unfavorable changes during this critical period of maturation may be achieved by endorsing breastfeeding and introduction of active protective compounds.
Nutrition to Endorse Compositional Development of Gut Microbiota

The mental impact of intrapartum antibiotic exposure appears to be most pronounced in infants born by cesarean section delivery [14, 15]. Indeed, long-term follow-up study shows that the impact of the mode of birth on gut microbiota composition may be intensified if broad-spectrum antibiotics were used during infancy. Neonatal antibiotic exposure has been reported to result in increased abundance of Proteobacteria and Firmicutes and reduced abundance of Bacteroidetes and Actinobacteria, particularly bifidobacteria, during the first weeks of life [15–18].

After birth, the sources of environmental exposure directly shaping the risk of disease are mainly associated with breastfeeding and, more specifically, the breast milk composition. Breast milk favors the predominance of bifidobacteria in the infant gut. Undeniably, the most important step of the colonization process comprises a rapid succession by anaerobic genera such as Bifidobacterium, Eubacterium, Clostridium, and increases in Bacteroides species. Different species of Bifidobacterium can reach up to 90% of the total fecal microbiota in breastfed infants, and frequently the composition comprises B. breve, B. infantis, and B. longum species, whereas the most common Lactobacillus in breast- and formula-fed infant feces are usually related to the Lactobacillus acidophilus group. Breastfeeding also exposes the infant to the mother’s skin bacteria. Recent study has shown the mean contribution of breast milk and areolar skin to the infant microbiome is 27.7% (SD 15) and 10.3% (SD 6.0), respectively [19]. Perez et al. [20] have shown that some bacterial signatures in breast milk are common to the infant’s feces and mother’s blood samples, which would imply a link. Hence, the breast milk microbiota contains a distinct bacterial community from skin, gut, vagina, or mouth.

A major step in the gut colonization coincides with weaning and subsequently towards microbial consortia characteristic of the adult microbiota by the age of 3 years [21, 22]. After weaning, the differences between breast- and formula-fed infants disappear due to the increase in the numbers of Bacteroides, Clostridium, and other anaerobic cocci in the former group, with general increases in numbers of E. coli and enterococci after weaning in both groups. During this step, rapid changes take place particularly in energy-harvesting Bacteroides species, this presumably reflecting the diet and the health of the host [21].

Following the first year of life, the rapid shift in microbiota composition and activity continues, and the community becomes more diverse, with Bacteroides, Veillonella, and Fusobacterium on the increase. At the same time, the number of unculturable microbes also increases, posing a challenge in characterizing the composition and the activity of the total microbiota. Nevertheless, it has been reported that children may still harbor higher numbers of bifidobacteria and enterobacteria than adults [21].
Optimal Nutrition for the Healthy Microbiome

The foundation of nutrition lies in a healthy, balanced diet to meet the needs for growth and development in children. Research interest in pediatric nutrition is currently directed beyond the nutritional impact of food towards the potential to reduce the risk of diseases, preferably benefiting from the concept of personalized nutrition. This is also the focus for microbiome research of specific active compounds with a documented capacity to strengthen the endogenous host defenses and to avert dysbiosis at an early age. However, the purpose is not to alter the gut microecology per se, but to adjust proinflammatory signals wired to the gut by the microbiota before altered structure and function in the target organ becomes consolidated (Fig. 1). The rationale is based on the model of a modern neonate, who is frequently exposed to unfavorable nutritional environment during pregnancy, born preterm or by cesarean section, or devoid of important immunomodulatory compounds of breast milk, and who thereby may lack an age-appropriate and environment-adjusted microbe contact. This in turn may substantially increase the child’s risk of developing noncommunicable diseases.

The Model Is the Healthy Breastfed Infant

The cornerstone of prevention of noncommunicable diseases is breastfeeding [23]. Not only does it provide the infant with nutrients, it may also confer immunologic protection at the portal of entry where the major load of antigens is encountered, the gut barrier [24]. A delicate balance of stimulatory, even inflammatory, maturational signals, together with a myriad of anti-inflammatory compounds, is transferred from mother to infant via breastfeeding. Human milk protective compounds also include specific oligosaccharides and fatty acids influencing early microbial colonization and gut barrier adherence of pathogens and other microbes, but also specific microbiota and molecules operating in host-microbe interaction.

Infants who have been breastfed have lower infectious morbidity and mortality, and protection against noncommunicable diseases has also been implicated [23]. Medicalization of breastfeeding, however, is both unwarranted and unattainable. Breastfeeding is the model of infant feeding even if beyond the ultimate medical proof; any documentation of causality requires well-controlled randomized clinical intervention studies in different human populations, which cannot be completed to assess breast versus formula feeding modes for obvious ethical reasons. Moreover, observational long-term follow-up studies in breast-versus formula-fed infants also face one important confounder: the evolution of the formula composition. Today’s formula composition in energy and nonenergy nutrient composition strongly differs from that of past decades.
The superiority of breastfeeding notwithstanding, infants who are exclusively breastfed may nevertheless develop allergic disease during breastfeeding or after weaning, or they develop other noncommunicable diseases later in life. This has been explained by the presence of antigens from the mother’s diet in breast milk or deficiency of key constituents in breast milk. In point of fact, the composition of the breast milk is not standard, but evinces marked individual variation, as a true product for personalized nutrition. The composition depends on environmental influences such as the mother’s immunological and metabolic health and the mode of delivery [25]. Moreover, early nutrition of the child needs to be considered when evaluating the long-term health effects of breastfeeding [26]. However, these comprise modifiable risk factors: the protective potential of breast milk and early nutrition can be enhanced as the science of diet, immune system, and microbiome interactions and the origins of human disease is evolving.

Determinants of Breast Milk Microbiota

The grounds for gut microbiota assembly may be generated already during the perinatal period. The mother provides the first inoculum of microbial colonization, possible already in utero (reviewed in the paragraph: Infant Gut Microbiota: Origin and Determinants of the Composition). There is abundant evidence that breast milk complements the microbiota transmission to the infant gut: the mother provides the infant with bifidobacteria, lactic acid bacteria, and other microbiota components in significant quantities during breastfeeding. Several active compounds of breast milk accomplish this progression. Among these, the breast milk microbiota profile is distinctive, reflecting the matrix of lipid and protein components. These components interact; the adhesion to mucosal surfaces, an important prerequisite of the immune-modulatory function of microbes, is modulated by polyunsaturated fatty acids.

Most of the bacteria isolated from breast milk belong to Staphylococcus and Streptococcus, followed by Lactobacillus and Bifidobacterium spp. [27]. Gut-associated strictly anaerobic microbes belonging to Blautia, Clostridium, Collinsella, and Veillonella and also some butyrate-producing bacteria such as Coprococcus and Faecalibacterium, as well as Roseburia have also been isolated in breast milk [28]. However, the new sequencing methodologies have provided evidence of a rich and diverse breast milk microbial community [28].

Above health-promoting microbes, human milk provides their optimal growth factors, human milk oligosaccharides, comprising over 200 prebiotic oligosaccharide isomers. Oligosaccharides typically pass undigested from the infant stomach and are the major nutrient source available for the saccharolytic microbiota of the colon. Indeed, variation in the oligosaccharide profile in milk
influences the microbial establishment in the infant gut. It has been reported that oligosaccharides favor the growth of specific gut bacterial groups such as *Staphylococcus* and *Bifidobacterium* spp. that also are present in breast milk [29]. The profile of human milk oligosaccharides has been described to affect infant gut microbial colonization by selectively promoting some bacteria and acting as decoy molecules for specific pathogens [30]. Infants fed by nonsecretor mothers, with lower presence of 2′FL (2′-fucosyllactose) exhibit delayed *Bifidobacterium* colonization and harbor lower numbers of *Bifidobacterium* than infants receiving breast milk from secretor mothers with higher abundance of 2′FL [31].

The microbes in breast milk strongly vary according to the mother’s health and weight gain during pregnancy [32]. To take an example, infants solely breastfed by their allergic and skin-prick-test-positive mothers had lower numbers of bifidobacteria than nonallergic mothers [33]. Similarly, *Bacteroides* and *Staphylococcus* were found to be significantly higher while *Bifidobacterium* counts were lower among gut microbes of overweight pregnant women and women with excessive weight gain during pregnancy than those with normal weight gain, and this distinction was reflected in the breast milk microbiota [32].

The mode of delivery has an influence on breast milk microbiota composition; distinct profiles have been documented between mothers delivering vaginally compared to those undergoing cesarean section delivery, but also between the types of cesarean delivery, i.e., elective versus nonelective cesarean section [25, 34], pointing to an impact of the physiological labor process, stress, and/or hormonal signals on the microbiota composition. In general, higher microbial diversity and abundance of *Bifidobacterium* and *Lactobacillus* characterize the breast milk microbiota of mothers after vaginal deliveries as compared to those delivering by cesarean section, but not consistently in different populations [34–36].

Importantly, it appears that *Bifidobacterium* colonization frequencies and counts among mother-infant pairs correlate. Moreover, the impact of the gut microbiota on the mucosal immune system evolution is well documented as a strain-dependent property [37]. Taken together, the infant’s probability of being colonized by bifidobacteria is lower when the mother has a higher BMI, excessive weight gain during pregnancy, and the child is delivered via cesarean section, and higher when the mother is of normal weight, has noticeable bifidobacterial colonization in her own gut and breast milk, and is breastfeeding.

**Bridging Early Nutrition to Health by the Microbiota**

The gut microbiota holds a key position with regard to the increasing burden of noncommunicable diseases in the industrialized countries. Its composition is relevant to the risk of disease in the gastrointestinal tract: the interaction be-
tween microbes and mucosal innate and adaptive immune systems provide the basis for achieving a halt to the vicious circle of inflammation therein. Recent advances in clinical research have revealed that the gut microbiota has effects on host physiology and development also outside the gastrointestinal system.

Our intervention studies with long-term follow-up corroborate the fundamental value of the gut microbiota profile at an early age to later health [38–40]. The studies found that children later developing allergic manifestations or becoming overweight had lower counts of bifidobacteria at the ages of both 6 and 12 months as compared to those remaining healthy, as well as a lower total *Bifidobacterium* genus pool, specifically of *B. longum* and *B. breve*. Moreover, administration of specific probiotics, compared to placebo, during the perinatal period and early infancy enabled to reduce the risk of allergic diseases throughout childhood until adolescence. Importantly, according to the multivariate logistic regression model, a lower risk of overweight was associated with breastfeeding duration ≥6 months compared to shorter duration.

Breastfeeding provides several health benefits [23] that are likely to be caused by promotion of age-appropriate and environment-adjusted gut colonization. Both precocious and delayed maturation of the gut microbiota seems to carry untoward immune and metabolic consequences [41]. It is of note that there are simultaneously occurring developments during infancy such as maturation of the gut barrier functions, reduction in breast milk consumption, and introduction of solid foods, which all impact on the compositional development of gut microbiota. While bifidobacteria typify the gut microbiota of a healthy breastfed infant, it is evident that *Lactobacillaceae* and *Bifidobacteriaceae* decrease upon introduction of solid foods and the transition period to family foods [reviewed in 21], with gut microbial diversity and richness significantly increasing concomitantly.

In general, the Western diet with its high fat and energy content has been associated with reduced gut microbiota diversity and perturbed composition, an imbalance in the taxonomic composition of the gut microbiota characterized as dysbiosis [42]. Reciprocally, the gut microbiota impacts on metabolism by retrieving nutrients otherwise inaccessible to the host; specific gut microbiota profiles facilitate the extraction of calories from the diet and their storage in the host adipose tissue [43]. Additionally, active inflammatory cascades evolve reactive to a high-fat diet. Interestingly, dietary fatty acids and microbes engage the same signaling pathways, linking the nutritional environment to the gut microecology within the innate immune regulation [11, 44]. Specifically, increased *Proteobacteria* have been considered markers or “signatures” of intestinal dysbiosis [45] while *Akkermansia muciniphila*, a member of Verrucomicrobia, appears to correlate inversely with inflammation [46]. Based on an American study, the con-
sumption of processed food was associated with a daily exposure to $10^6$ bacteria as compared to a diet recommended by the dietary guidelines ($10^9$ bacteria) [47], thus demonstrating the decrease in richness.

The strong association between early nutrition and the compositional development of the gut microbiota, both impacting on the individual’s later health, invite the idea of next-generation personalized diets based on specific risk algorithms. These systems would certainly benefit from rapid diagnostics of the gut microbiota profiles. Indeed, it has been proposed that the gut microbiota provides the key determinant to be considered when developing specific dietary products for the need of both developing and developed countries [6, 41].

Taken together, dysbiosis is a necessary initial step in the development of noncommunicable diseases on the one hand and undernutrition on the other. An attractive vision arising from recent experimental and clinical studies is to identify and target disease risk by bringing the gut microbiota into balance. Re-programming at an early age may necessitate well-adjusted age-appropriate food matrix for active compounds with scientifically proven safety and efficacy assessment, and the optimal timing of the intervention before consolidation of target organ dysfunction.

Disclosure Statement

Nothing to disclose.

References


Human Milk and Clinical Outcomes in Preterm Infants

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Abstract
The LOVE MOM cohort (Longitudinal Outcomes of VLBW Infants Exposed to Mothers’ Own Milk; NIH: R010009; Meier PI) enrolled 430 infants with very low birth weight (VLBW) between 2008 and 2012 to study the impact of the dose and exposure period of MOM during hospitalization in the neonatal intensive care unit (NICU) on potentially preventable complications of prematurity and their associated costs. In this prospective study, MOM and formula feedings were calculated daily (mL), medical diagnoses for NICU morbidities (necrotizing enterocolitis [NEC], late-onset sepsis [sepsis], and bronchopulmonary dysplasia [BPD]) were confirmed independently by 2 neonatologists, and propensity scoring was used to analyze covariates. Neurodevelopmental outcome was measured for a subset of 251 LOVE MOM infants at 20 months of age, corrected for prematurity (CA). Data revealed a dose-response relationship between higher amounts of MOM received during critical NICU exposure periods and a reduction in the risk of NEC, sepsis, BPD, and their costs, as well as higher cognitive index scores at 20 months CA. MOM appears to function via different mechanisms during NICU exposure periods to reduce the risk of potentially preventable complications and their costs in VLBW infants. Institutions should prioritize the economic investments needed to acquire, store, and feed high-dose MOM in this population.

Introduction
Historically, human milk (HM; includes mother’s own milk [MOM] and donor HM [DHM]) was prioritized for the feeding of premature infants throughout the world [1, 2]. However, the first actual research comparison of HM and non-HM was a case-control study that evolved from the observation that retrolental fibro-
plasia, known today as retinopathy of prematurity, seemed to occur less frequently in premature nurseries where HM feedings were used [3]. In the ensuing decades, there was little interest in the scientific study of clinical outcomes of HM feedings in premature infants, and the primary reason to support MOM feeding in the neonatal intensive care unit (NICU) was to support maternal involvement in infant care. The state of this science was changed completely in the mid-1980s with the study by Lucas et al. [4] who randomized 926 premature infants to different feeding interventions including MOM, DHM, and preterm and term formulas. Known as the Lucas cohort, participating infants were studied during the initial hospitalization, infancy, childhood, and young adulthood, resulting in the most comprehensive set of clinical outcome data on the impact of MOM, DHM, and formula feeding for premature infants available at that time [4, 5]. Their primary hypothesis about the role of early nutrition in the programming of these outcomes is still relevant today [6]. Clinically, these data spearheaded changes in NICU best practices, with a new focus on encouraging mothers to provide their MOM, even though their initial intent may have been to formula feed [7].

In the past 20 years, multiple studies have reported that MOM feedings reduce the risk of potentially preventable complications of prematurity that occur during NICU hospitalization, including necrotizing enterocolitis (NEC), late-onset sepsis (sepsis), and bronchopulmonary dysplasia (BPD) [8]. These morbidities significantly increase the risk for subsequent neurodevelopmental problems and other chronic health conditions throughout childhood, as evidenced by the high rates of rehospitalization and special education needs in this population [8, 9]. Furthermore, NEC, sepsis, and BPD significantly increase the marginal costs of NICU care that are attributable both to prolonged length of the NICU hospitalization and to greater resource use when compared to infants who do not acquire these morbidities (Fig. 1) [10, 11]. Although this body of research suggested a cost-benefit to the feeding of high-dose MOM during NICU hospitalization, the individual studies were characterized by multiple methodological inadequacies (Table 1), limiting their ability to inform evidence-based NICU practice. In the absence of rigorous and compelling research about the effectiveness of MOM in reducing these serious and costly morbidities, hospital administrators were reluctant to invest economically in the NICU infrastructure required for the acquisition, storage, and feeding of MOM.

**LOVE MOM Cohort**

The LOVE MOM (Longitudinal Outcomes of VLBW Infants Exposed to Mothers’ Own Milk) cohort enrolled 430 very low birth weight (VLBW) infants (95% of eligible subjects) between 2008 and 2012 to study the relationship between the
dose and exposure period of MOM feedings during NICU hospitalization and the risk of NEC, sepsis, and BPD, and their associated NICU costs. (National Institutes of Health, NR010009, Meier, PI). This prospective cohort study was designed to address the limitations in Table 1, with the exception of the inability to randomize to feeding groups.

Central to the conceptualization of this study was determining which dose of MOM over which critical exposure periods during the NICU hospitalization reduces the risk of the specific morbidity. These morbidities occur at different ages after birth, are multifactorial, and may be differentially affected by specific MOM mechanisms or components [12]. For example, VLBW infants are at the greatest risk for NEC during the very early period after birth, and a key mechanism in the pathogenesis of NEC is local inflammation in the gut epithelial border [13]. Thus, we speculated MOM may reduce NEC via the array of growth factors, anti-inflammatory components, and other protective components in the early MOM produced by mothers who deliver prematurely [8]. Similarly, when modeling the effect of MOM on these morbidities, there is potential for reverse causality in which the dose of MOM is reduced due to the presence of the morbidity. For example, an early-onset morbidity such as NEC is managed clinically by placing the infant NPO (nil per os; not being fed enterally), so MOM

**Fig. 1.** Marginal costs of morbidities by birth weight, adjusted for infant sociodemographic characteristics [10, used with permission].
Table 1. Limitations in research addressing the impact of MOM feedings on health outcomes for premature infants

<table>
<thead>
<tr>
<th>Limitations</th>
<th>Impact on findings</th>
<th>LOVE MOM cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inability to randomly assign infants to feeding groups</td>
<td>Self-selection bias, e.g., factors associated with the choice and/or ability to provide MOM affect the outcome variable</td>
<td>Prospective cohort design with dose-response methodology 95% of eligible infants enrolled</td>
</tr>
<tr>
<td>Inconsistent definition of subjects (premature, extremely premature, LBW, VLBW, ELBW)</td>
<td>Different stages of infant maturity (and, thus, susceptibility to potentially preventable morbidities) make generalization of study findings difficult Findings from studies of larger, healthier populations likely underrepresent beneficial outcomes for less mature and/or sick infants</td>
<td>Enrolled only VLBW infants Subanalyses permitted birth-weight-specific (e.g., ELBW vs. VLBW) analyses for relevant morbidities</td>
</tr>
<tr>
<td>Lack of inclusion of MOM-fed infants born to minority and/or low-income mothers</td>
<td>Self-selection bias that limits generalization of findings to the most vulnerable infants, potentially underrepresenting MOM impact</td>
<td>52% of cohort = Black 27% of cohort = Hispanic 70% of cohort = low-income</td>
</tr>
<tr>
<td>Inconsistent definition and measurement of MOM feeding</td>
<td>Estimates of dose and exposure period of MOM are imprecise, especially in secondary analyses and retrospective studies MOM and DHM are frequently combined into a single HM metric, underrepresenting the impact of MOM Reverse causality is possible if the MOM exposure period in the NICU is not described (i.e., Infant placed NPO due to morbidity, and NPO decreases MOM exposure)</td>
<td>MOM feeding measured prospectively to the nearest mL Defined as weight-adjusted dose and percentage of total enteral feedings Exposure period measured for 1–14 and 1–28 days of life, entire NICU hospitalization, and at 36 corrected weeks of gestation (for BPD modeling)</td>
</tr>
<tr>
<td>Inconsistent and/or inaccurate diagnoses of medical outcomes (NEC, clinical vs. culture-proven sepsis)</td>
<td>Lack of use of standardized definitions compromises internal validity of findings, especially for NEC (vs. spontaneous perforation) and sepsis (clinical vs. culture proven) Use of combinations of morbidities in a single primary outcome measure (risk of NEC, sepsis, or death) to compensate</td>
<td>Medical diagnoses prospectively recorded in “real time,” not retroactively from patient records Standardized, prospectively defined, medically accepted criteria used for diagnoses All diagnoses confirmed independently by 2 neonatologists</td>
</tr>
<tr>
<td>Relatively low rates of morbidities for which risks are multifactorial, limiting the statistical analyses to test the impact of MOM feeding in the absence of very large samples</td>
<td>Either important covariates not addressed or Effect of MOM not significant because of too many covariates and small sample size or Several morbidities are combined into single primary outcome measures (NEC, sepsis, or death) to offset low rates of individual morbidities</td>
<td>Use of propensity scoring to combine multiple covariates into a single score Propensity scoring technique then assigned a proxy score to each infant in the dataset based upon the probability that the infant would develop the morbidity under study</td>
</tr>
<tr>
<td>Hospital charge or cost-to-charge ratio data are imprecise, inaccurate and vary with the institution</td>
<td>Variability among institutions make data difficult to interpret and generalize</td>
<td>Use of actuarial cost data from the hospital accounting system allowed precise measurement of costs and allocation of costs to specific categories (e.g., nursing care, radiology, pharmacy, etc.)</td>
</tr>
</tbody>
</table>

NOP, nil per os.
is not fed during this time. If, as the independent variable, MOM is measured after the morbidity occurs, the low MOM intake may be a result of the morbidity versus a factor that contributed to its occurrence.

Although several studies had examined the contribution of MOM to neurodevelopmental outcome in premature, low birth weight (LBW), and extremely LBW (ELBW) infants (but not in VLBW infants) at the time the LOVE MOM cohort was created [11], follow-up through to 20 months of age, corrected for prematurity (CA) was not a part of the original NIH-funded study. Instead, the research on neurodevelopmental outcome in LOVE MOM was funded separately and included a subset of 251 smaller and sicker LOVE MOM cohort infants [14].

Subjects
LOVE MOM inclusion criteria were: birth weight <1,500 g; gestational age ≤35 weeks; if multiple birth, one infant selected randomly to participate; absence of congenital anomalies that might influence enteral feedings and/or cost of care; inborn or transferred to the Rush NICU within 24 h after birth; fed enterally within 14 days after birth; and negative maternal drug screen. Although maternal initiation of lactation was not an inclusion criterion, 98% of infants received some MOM. Table 2 summarizes the characteristics of the LOVE MOM cohort and their mothers. Unlike previous studies in this area, the sample consisted primarily of infants born to minority, low-income mothers who are the most likely in the United States to give birth to VLBW infants, but the least likely to initiate and maintain lactation [15].

Measures
The independent and dependent variables for this study have been detailed in original research papers and are summarized briefly here. All measures were collected prospectively and entered into the study database.

Dose and Exposure Period of MOM. Parenteral and enteral feedings were advanced according to a standardized protocol, and bovine-based powdered fortifier was added to MOM feedings when enteral feeding volume reached 140 mL/kg/day. No DHM was used during this study, so infants received either fortified MOM, formula, or a mixture of both. Colostrum was fed in the order produced by the mother through to the achievement of full enteral feedings; thereafter, fresh MOM was prioritized over frozen MOM [16–18].

During each day of the NICU hospitalization, the milliliters of MOM and commercial formula were summed. These figures were used to calculate both a weight-adjusted MOM daily dose (HM-DD; mL/kg/day) and the percent of enteral feedings equal to MOM (HM-PCT; mL MOM/[mL MOM + mL formula]). These MOM doses were calculated for 3 specific NICU exposure periods after
birth: first 14 days of life (DOL); first 28 DOL (including first 14 days); and the entire NICU hospitalization [12]. For modeling of BPD, MOM dose was also calculated when the infant reached 36 weeks postmenstrual age because BPD is diagnosed at this time point.

**Acquired Medical Morbidities.** Specific criteria used for the diagnosis of NEC, sepsis, BPD, and neurodevelopmental problems were consistent with standard definitions in the literature and are detailed in the original publications. Each medical diagnosis was confirmed independently by 2 neonatologists.

**NICU Cost of Care.** Institutional costs were calculated by summing the direct cost of care for each chargeable item using the institution’s system-wide cost accounting system and combining this figure with indirect costs to yield total institutional costs. Provider costs were analyzed separately, and total costs were adjusted to the year in which the original papers were published [16–18].

### Table 2. Characteristics of the LOVE MOM cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 430)</th>
<th>Black (n = 223)</th>
<th>White (n = 83)</th>
<th>Hispanic (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age, years</td>
<td>27.2±6.5</td>
<td>26.0</td>
<td>28.7</td>
<td>28.0</td>
</tr>
<tr>
<td>Maternal race/ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>223 (52%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>83 (19%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>114 (27%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10 (2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal education, years (n = 421)</td>
<td>13.2±2.8</td>
<td>13.3</td>
<td>14.6</td>
<td>11.7</td>
</tr>
<tr>
<td>Medicaid at most recent time point</td>
<td>306 (71%)</td>
<td>82%</td>
<td>47%</td>
<td>74%</td>
</tr>
<tr>
<td>Medicaid during NICU stay</td>
<td>269 (63%)</td>
<td>71%</td>
<td>34%</td>
<td>68%</td>
</tr>
<tr>
<td>WIC eligible (n = 425)a</td>
<td>298 (70%)</td>
<td>81%</td>
<td>33%</td>
<td>78%</td>
</tr>
<tr>
<td>Primigravida</td>
<td>143 (33%)</td>
<td>27%</td>
<td>45%</td>
<td>39%</td>
</tr>
<tr>
<td>Providing any HM to infant in NICU</td>
<td>420 (98%)</td>
<td>96%</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Infant characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple gestation</td>
<td>61 (14%)</td>
<td>10%</td>
<td>34%</td>
<td>7%</td>
</tr>
<tr>
<td>Male</td>
<td>229 (53%)</td>
<td>53%</td>
<td>58%</td>
<td>54%</td>
</tr>
<tr>
<td>Gestational age, completed weeks</td>
<td>28.0±2.4</td>
<td>27.9</td>
<td>27.6</td>
<td>28.2</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>1,046±256</td>
<td>1,030</td>
<td>1,008</td>
<td>1,103</td>
</tr>
<tr>
<td>Birth: small for gestational age</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(below 10th percentile)</td>
<td>86 (20%)</td>
<td>20%</td>
<td>19%</td>
<td>18%</td>
</tr>
<tr>
<td>Length of NICU hospitalization, days</td>
<td>73.8±41.9</td>
<td>75</td>
<td>79</td>
<td>69</td>
</tr>
<tr>
<td>Discharge weight below 10th percentile</td>
<td>153 (36%)</td>
<td>51%</td>
<td>22%</td>
<td>24%</td>
</tr>
<tr>
<td>Death</td>
<td>7 (2%)</td>
<td>3%</td>
<td>1%</td>
<td>0%</td>
</tr>
</tbody>
</table>

n (%) or means ± SD.

a WIC-eligible; Supplemental Security Income, Women, Infant and Children eligibility; <185% of federal poverty level; measure of low-income.
Statistical Approaches

The overall incidence of these acquired morbidities is low, limiting the statistical analyses that can be used to model the risk of the morbidity as a function of MOM feedings. In the United States, NEC occurs in 5–7% of VLBW infants; sepsis in 22%, and BPD in 26–33% [19]. Additionally, these morbidities are considered multifactorial with numerous covariates including infant birth weight, gestational age, gender, duration of total parenteral nutrition, the day that enteral feedings were initiated after birth, use of prophylactic antibiotics beyond 48 h after birth, blood transfusion, ventilatory requirements, and others factors specific to the morbidity. Thus, controlling for the effect of these covariates on the outcome morbidity with a reasonable sample size has been a problem throughout MOM research with this population [20]. Our study incorporated propensity scoring to combine the covariates for each morbidity into a single risk score, which was then applied to each infant in the dataset as a proxy variable for the combination of covariates [21]. This technique permitted modeling the risk of the morbidity with a reasonable sample size, while controlling statistically for covariates.

Results

A dose-response relationship was demonstrated between the dose of MOM and a reduction in the risk of NEC, sepsis, BPD, and neurodevelopmental problems, with higher doses of MOM translating into lower risks and associated costs for the specific morbidity [14, 16–18]. Each morbidity and its associated cost reduction was linked to high-dose MOM during a specific critical exposure period during the NICU hospitalization, as detailed in Table 3.

Discussion

To the best of our knowledge, this is the first prospective study to report both the health and cost outcomes of MOM feedings in a contemporary, heterogeneous cohort of VLBW infants during the NICU hospitalization and again at 20 months CA. Cumulatively, these 4 publications reveal a dose-response relationship between the volumes of MOM received during critical exposure periods in the NICU and a reduction in the risk of the specific morbidity and its associated costs. Furthermore, our 20-month data indicate that high-dose MOM feedings through to NICU discharge significantly decreased the risk of neurodevelopmental problems in a subset of the smallest, sickest infants in the LOVE MOM cohort.
Necrotizing Enterocolitis

Three separate studies, a prospective [22], a retrospective [23], and a secondary analysis of an existing dataset [24], have reported similar findings about the reduction in NEC with high-dose MOM feedings during the early period after birth. In a prospective study, Sisk et al. [22] reported that odds of NEC were decreased 6-fold in VLBW infants who received ≥50% of their enteral feed volume as MOM during the first 14 DOL. In a retrospective analysis of 349 VLBW infants, Corpeleijn et al. [23] reported similar findings for the percentage of MOM received during the first 10 DOL and a reduction in the combined variable of NEC, sepsis, or death. In a secondary analysis of data from 1,272 ELBW infants enrolled in the NICHD-funded multisite glutamine trial, Meinzen-Derr et al. [24] reported a dose-response relationship between the percentage of enteral feedings equal to MOM during the first 14 DOL and a reduction in the risk of either NEC or death after 14 DOL, with a trend towards the greatest reduction being afforded by 100% MOM feedings.

Whereas multiple bioactive components in early MOM colostrum and transitional milk provide support for growth, protection and maturation of the vulnerable gut epithelial border during the transition from intra- to extrauterine
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Sepsis
Similar to our findings, Furman et al. [30] reported a dose-response relationship between the dose of MOM feedings (≥50 and 25–49.99 mL/kg/day) received during the first 28 DOL and reduction in sepsis for VLBW infants. Whereas multiple other studies have reported a reduction in the risk of sepsis with MOM feedings, major limitations are whether sepsis was measured as clinical or culture-proven and whether the potential for reverse causality was addressed [31].

The findings that MOM feedings do not need to be exclusive in order to reduce the risk of sepsis, and that a lesser dose of 25–49.99 mL/kg/day of MOM affords some protection suggest that the bioactive components in MOM rather than the avoidance of bovine-based products is a major mechanism of protection. This speculation is supported by the findings that DHM affords no reduction in the risk of sepsis in this population [26]. Likely bioactive components include anti-infectives such as secretory IgA, a MOM proteome that targets immunomodulation, MOM-borne probiotic bacteria, and oligosaccharides with prebiotic activity [16, 25]. Additionally, sepsis is linked with multiple factors that are indirectly affected by MOM feedings such as shorter duration of central-line catheters and parenteral nutrition, both of which increase the risk of sepsis [16].

Bronchopulmonary Dysplasia
Only one previous multicenter cohort study has compared the rates of BPD for exclusively HM-fed (mostly MOM with some DHM; n = 223) and exclusively formula-fed (n = 249) VLBW infants, reporting significantly lower rates of BPD in exclusively HM-fed infants [32]. These findings are remarkably similar to ours, with the exception that Spiegler et al. [32] compared only the two extremes of MOM dose (exclusive or none), whereas we reported a dose-response relationship with each additional 10% of enteral feedings between birth and 36 weeks postmenstrual age reducing the odds of BPD by 9.5%. Of particular interest is that both studies found slightly slower rates of in-NICU weight gain with
high-dose MOM feedings, suggesting that there may be a trade-off between reducing BPD and achieving weight gain targets in VLBW infants [18, 32].

Although BPD is much less studied than other potentially preventable morbidities, MOM feedings provide recipient infants with antioxidant [33] and anti-inflammatory [34] components as well as myoinositol [35], which has been linked with a reduction in BPD when used as a supplement in this population [36]. Additionally, MOM may provide protection from BPD indirectly by reducing the risk of NEC and sepsis, both of which upregulate inflammation and have been associated with the subsequent development of BPD [18].

**Neurodevelopmental Outcome at 20 Months Corrected Age**

Previous studies have examined the relationship between MOM feedings and neurodevelopmental outcome in premature infants, but all have been characterized by the multiple limitations outlined in Table 1 [11]. To our knowledge, only our study included meticulous prospectively collected information about the NICU dose and exposure period of MOM feedings in a contemporary cohort of VLBW infants. Our findings revealed a significant dose-response relationship, with each additional 10 mL/kg/day MOM intake during the NICU hospitalization associated with an additional 0.35 cognitive index score. This difference is equal to a 5-point difference between our lowest and highest MOM dosing groups, despite the fact that infants in the highest quintile of MOM dose gained weight slightly less rapidly and were more likely to be discharged below the 10th percentile for weight (extrauterine growth retardation; EUGR) than infants in lower MOM dosing quintiles. In bivariate analyses, EUGR was a significant risk factor for neurodevelopmental problems, but in multivariate analyses EUGR was mitigated by high-dose MOM feedings. In other words, if the slightly slower weight gain during the NICU hospitalization is a function of high-dose MOM feedings, our findings suggest that high-dose MOM feedings should be prioritized.

In addition to providing nutritional substrate to optimally support the high metabolic activity and growth of the human brain, bioactive MOM components provide neuroprotection to the vulnerable, rapidly growing white matter in the premature infant brain, the integrity of which has been linked to subsequent neurodevelopmental outcome in several studies [5, 37–40]. Furthermore, NEC, sepsis, and BPD increase the risks of neurodevelopmental problems in premature infants, so the fact that MOM feedings reduce these NICU morbidities contributes indirectly to neurodevelopmental outcome differences [14]. In a large randomized trial of MOM feedings supplemented with either DHM or formula in VLBW infants, DHM feedings had no beneficial impact on neurodevelopmental outcome [41].
Conclusion

High-dose MOM feedings during 3 critical exposure periods in the NICU hospitalization provide significant reductions in the risk of NEC, sepsis, BPD, and neurodevelopmental problems in VLBW infants. These same outcomes are not achievable with either DHM or infant formula. Furthermore, the reduction in these morbidities translates into significant cost savings, and findings support the economic investment into the NICU infrastructure that is necessary for the acquisition, storage, and feeding of MOM.

Disclosure Statement

This work was funded with a grant (NR010009) from the National Institutes of Health. No conflicts of interest to declare.

References


In this session, the presenters considered the impact of human milk on clinical outcomes of term and preterm infants, incorporating aspects of growth and metabolic outcomes and cognitive, immune, and microbiome development.

Ferdinand Haschke kicked off the session by reviewing the role of breastfeeding and human milk in long-term programming of growth, metabolic outcome, and health in three populations of infants at risk for later-life morbidity. The first are very-low-birthweight (VLBW) infants who receive fortified human milk. For VLBW who are also born too small, ESPGHAN recommends providing extra nutrients up to 52 weeks of postconceptional age [1]; however, it was not known if different nutritional regimens were needed for VLBW infants who were small for gestational age (SGA) with no genetic defects, malformations, intrauterine infections, versus VLBW infants who are intrauterine growth retarded (IUGR) with pathological ultrasound measurements. Ferdinand Haschke reviewed the findings of the clinical trials demonstrating that VLBW SGA infants discharged on fortified human milk grew better than did IUGR infants who showed a decrease in weight percentiles between discharge and 3 months corrected gestational age [2]. Their findings suggest that different nutrition guidelines for VLBW who are SGA or IUGR may be warranted; however, randomized controlled studies with larger populations of infants are needed. The second population are infants from developing countries who are breastfed, but have low birthweight and length. Demographic health survey data from 20 developing countries were used to assess the longitudinal growth patterns of
>130,000 children at intervals between birth and age 2 years [3]. Exclusive breastfeeding was associated with significantly higher weight, length, and lower probability of stunting, wasting, and infections. Growth faltering observed between 6 and 24 months likely reflected the low-quality complementary feedings and poor environmental conditions in developing countries. The importance of maternal health and nutritional status prior to conception and during pregnancy for infant growth was highlighted by the observation that infants born in the lowest 10th percentile exhibited poor growth and severe stunting by age 2 years, whereas infants in the top 10th percentile tracked along the 50th percentile on the WHO growth curve. Thus, holistic interventions should encompass maternal nutritional status, early-life nutrition (exclusive breastfeeding), and high-quality weaning foods. The last population are children from developed countries who were enrolled in randomized controlled trials (RCTs) to test if breastfeeding and low-protein formulas can prevent rapid weight gain and childhood obesity. Infant formula has traditionally contained a higher protein content than human milk. Longitudinal RCTs demonstrate that breastfeeding and the use of low-protein formulas prevent rapid weight gain during infancy and, ultimately, the risk of childhood obesity [4]. A recent French cohort study showed that protein intake (>15% of calories) was associated with higher BMI during school age, adolescence, and young adulthood [5]. While human milk is the desired form of nutrition for all infants, these studies suggest that its long-term effects on infant growth are influenced by the infant’s physiological state (term vs. VLBW) and environment (developing vs. developed country).

Weili Lin described the pre- and postnatal stages of brain development and the evidence linking early-life nutrition to brain and cognitive development in human infants. The potential actions of dietary docosahexaenoic acid, iron, choline, and sphingomyelin were discussed in terms of critical windows of exposure and mechanisms of action. He next discussed behavioral and cognitive assessments that have been used to assess neurodevelopmental outcomes in human infants and their limitations (e.g., they do not provide a direct assessment of the neural substrates underlying brain functional maturation). To overcome this limitation, Weili Lin and colleagues utilize magnetic resonance imaging to non-invasively image the brains of breast- and formula-fed infants during early life. He highlighted some preliminary findings of the Baby Connectome Project – Enriched study (BCP-Enriched), a study specifically designed to discover the complex interaction of nutrition, the gut-brain axis, and early brain maturation. The study should offer invaluable insights into the interaction of these essential physiological parameters. This knowledge has the potential to guide recommendations on nutrient supplementation during critical periods in early childhood to promote the healthy brain growth in early life.
Valerie Verhasselt summarized the importance of early-life nutrition and gut immune development. Defects in the development of tolerance and mucosal immune regulation are implicated in increased risk of allergy and obesity-associated diseases. Thus, understanding the factors modulating gut immune development could inform primary prevention strategies specifically adapted to the early-life immune system. She first reviewed gut mucosal immunity development and the role of early nutrition in mediating this process. She reviewed the emerging evidence for the important role of dietary antigens, vitamin A and Aryl hydrocarbon receptor ligands in early-life immune development and function, and the potential for human milk to be a delivery vehicle for these components. Valerie Verhasselt also discussed the importance of early-life nutrition in shaping the composition of the gut microbiota, which in turn affect immune development and the gut-brain axis.

Building on the previous two talks, Erika Isolauri’s presentation focused on early-life nutrition and the development of the microbiome. Dysbiosis has been linked to a wide-range of clinical conditions ranging from inflammatory bowel disease, obesity, and allergic diseases to neurodevelopmental disorders. There is a growing interest in defining what constitutes a healthy microbiome, how it interacts with the host and whether it can be modified by diet. Erika Isolauri provided an overview of factors influencing the acquisition of the gut microbiota in early life, including route of delivery, form of nutrition, weaning, and antibiotic administration to the mother and infant. Human milk is considered the gold standard for human infants, and the microbiota of the breastfed infant differs from that of formula-fed infants. This difference is due in part to the presence of a human milk microbial consortium and human milk oligosaccharides that serve as prebiotics for the infant microbiome. While our understanding of the development of the microbiome has advanced over the past decade, a more complete understanding of the mechanisms underlying immune-mediated and microbiome-associated chronic conditions is needed. In addition, she highlighted the need for rigorous clinical intervention studies with long-term follow-up to determine whether early-life differences in the microbiota are causally linked to the pathogenesis of microbiome-associated diseases.

In the final talk of the session, Paula Meier focused on human milk feeding for preterm infants in terms of type of feeding – mother’s own milk (MOM) and donor human milk, health outcomes, and economic benefits of feeding MOM. Paula Meier described the findings of the NIH-funded LOVE MOM (Longitudinal Outcomes of VLBW Infants Exposed to Mothers’ Own Milk) cohort that studied the relationship between the dose and exposure period of MOM feedings during hospitalization in the neonatal intensive care unit (NICU) and the risk of necrotizing enterocolitis (NEC), sepsis and bronchopulmonary dysplasia.
(BPD), and their associated NICU costs. They hypothesized that a dose of MOM over a specific critical exposure period would reduce the risk of a specific morbidity, with the knowledge that these morbidities occur at different postbirth ages, are multifactorial, and may be differentially affected by specific MOM mechanisms or milk components. They demonstrated dose-dependent relationships, with higher doses of MOM being associated with lower risks of NEC, sepsis, and BPD. Thus, MOM has the potential to improve the short- and long-term health outcomes of preterm infants and reduce the healthcare costs associated with caring for these high-risk infants.

Sharon M. Donovan

References

Research Gap and Opportunities


Metabolomics in Human Milk Research

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Abstract

The link between food and health is complex, particularly for the developing neonate, as the period after birth is the time when long-term programming is occurring notably in the neurologic, immune, and metabolic regulatory systems. Breastfeeding is known to have short- and long-term benefits, and yet the intricate relationship of this unique food with the neonate is not fully understood. Application of multi-omic approaches incorporating new bioinformatic tools will allow for better characterization of phenotypes over the traditional approaches that were limited to crude assessment of growth parameters and observation of clinical disease. Metabolomics has the capability of allowing for a relatively noninvasive assessment of phenotypes via the assessment of small molecules in biofluids such as serum or urine that provides an opportunity to assess metabolism systemically in the developing neonate. Metabolomics can also be used to assess the metabolic activities of gut microbes through measurement of microbial by-products in the stool. Understanding the composition of human milk, how its components work synergistically together, and how they change over time will provide insight into how immunity and metabolism is established in early life, and how it can potentially prevent the development of chronic diseases in later life.

Introduction

The postnatal period for human infants is recognized as a key period for growth and development, particularly for the rapidly developing brain. All of the infant’s metabolic, immunologic, intestinal, and physiologic systems are also rap-
idly changing. The ability of milk to nourish, fuel, and supply substrates to this growth has been recognized [1]. However, the role of milk in orchestrating the entire metabolic process is not clear. A new generation of tools are now available to interrogate both milk and its effects on infant metabolism. Here, metabolomics is defined as the science of analyzing metabolites as an ensemble with sufficient diversity to map those metabolites onto pathways and sufficient quantitative accuracy to estimate metabolite flows through those pathways.

Brain growth during infancy has been positively associated with intelligence [2, 3], and human milk helps promote that development, particularly white matter development [4]. Indeed, the human brain volume increases between 8 and 15% every 3 months from birth until approximately 18 months of age. Brain metabolites also change with dramatic increases in N-acetylaspartate, creatine, and glutamate and decreases in myoinositol in the cerebral white matter and cortex during the first 3 postnatal months [5]. Epidemiological evidence suggests that breastfeeding practice is positively associated with intelligence and educational attainment at age 30 [6], and negatively associated with obesity, cardiovascular disease [7], and diabetes [8] in adulthood. How human milk is able to fuel and shape metabolism and guide overall health and development is an ongoing area of active research, and clues are beginning to emerge that the microbiota within the infant gastrointestinal (GI) tract are important.

The period immediately after birth, and for the first 2–3 years of the child’s life, is a key period for the development of intestinal microbiota [reviewed in 9, 10]. In the GI tract resides an ecosystem where microbes must compete in order to survive and persist, and the host must shape the microbiota in order to foster a beneficial community [11]. One theory states that for symbiont-directed control, where a microbe alters global host phenotype to increase its own fitness, there must be low microbial diversity and limited competition between microbes [11]. Interestingly, until the weaning period, breastfed infants have lower microbial diversity than formula-fed infants [reviewed in 12]. Infancy is also a time of instability with respect to the gut microbiota as evidenced by greater interindividual variability compared with adults [reviewed in 13]. This instability represents an important window of opportunity for the development of the gut microbial community. A recent study using gnotobiotic mice demonstrated that there is a critical window of time for intestinal immune development, and if conventionalization does not occur during that period, immune development cannot be fully achieved [14]. Evidence is accumulating that the immune system is linked inextricably with global metabolism [15]. Thus, it stands to reason that immune development will have important consequences for metabolic development [16], and if both are not optimally established early in life, consequences may be realized later in life.
The complex nature of milk therefore means that it has important functions for optimizing the health and the microbiota of the infant. Understanding the intricacies of the system at each stage of childhood that are vital for establishing the eventual host phenotype, including immunity, metabolism, and brain health, will be important for understanding the role of milk and its components in shaping health. While we are not yet at a stage where these complex relationships have been elucidated, linking host genetics and microbial ecology with the environment, including diet (macro- and micronutrient composition), and phenotype (measured through metabolomic analysis of serum, urine, and feces) will help us move toward that goal.

**Human Milk and the Human Milk Metabolome**

During the first few months of life, human milk provides essential nutrients for the infant while helping to establish the bacterial species that will constitute the gut microbiome. In addition to providing the essentials for growth and development, proteins, fatty acids, carbohydrates, and micronutrients, human milk provides a variety of cytokines, inflammatory mediators, and signaling molecules [reviewed in 17, 18].

Human milk varies greatly throughout lactation, with the colostrum very rich with immune factors and oligosaccharides that tend to decrease as lactation progresses [reviewed in 17]. Interestingly, we and others [19–23] observed a number of metabolites that change in concentration over time in human milk that includes amino acids, sugars, fatty acids, and others (Table 1). In general, oligosaccharides tend to decrease over time, while increases in lactose, several amino acids, as well as short- and medium-chain free fatty acids are noted. What this means is that milk maturation is not stochastic. Its composition is carefully controlled by the mammary gland that is guided by maternal genetics, continuously changing to meet the nutrient requirements of the neonate and help guide microbial succession throughout the period of exclusive milk feeding and possibly beyond. It is interesting to point out that microbial and fecal metabolic profiles are more similar between formula-fed infants from different mothers than breastfed infants from different mothers [24, 25], which could be attributed to the differences in milk composition between mothers who are breastfeeding and driven by changes in milk composition over the lactation period. In contrast, the composition of formula does not change. These results imply that diet (breast- or formula feeding) is a key driver in selecting microbes to colonize the GI tract.

One example of how maternal genetics tightly controls milk composition may be observed through the correlation of 2 of the more abundant oligosaccharides: 2′-fucosyllactose (2′FL) and 3-fucosyllactose (3FL). The concentration of these two oligosaccharides is dictated by the expression levels of enzymes encoded by the fucosyltransferase 2 (FUT2) and fucosyltransferase 3 (FUT3) genes, both of
Table 1. Variation in milk metabolites over time in women who are secretors

<table>
<thead>
<tr>
<th>Metabolites increasing over lactation</th>
<th>Metabolites decreasing over lactation</th>
<th>Metabolites with no significant trend</th>
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<tbody>
<tr>
<td><strong>Sugars</strong></td>
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<tr>
<td>Lactose</td>
<td>2'-Fucosyllactose</td>
<td>Galactose</td>
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<td>3'-Fucosyllactose</td>
<td>3'-Galactosyllactose</td>
<td>Lactodifucotetraose</td>
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<td>Glucose</td>
<td>3'-Sialyllactose</td>
<td>Lacto-N-fucopentaose II</td>
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<td>6'-Sialyllactose</td>
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<td>Fucose</td>
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<td>Lacto-N-tetraose</td>
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<td>Lacto-N-fucopentaose I</td>
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<td>Lacto-N-fucopentaose III</td>
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<td>Sialic acid</td>
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<td></td>
<td>Myo-inositol</td>
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<td><strong>Amino acids</strong></td>
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<td>Alanine</td>
<td>Leucine</td>
<td>Asparagine</td>
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<td>Glutamate</td>
<td>Lysine</td>
<td>Aspartate</td>
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<td>Glutamine</td>
<td>Proline</td>
<td>Histidine</td>
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<td>Phenylalanine</td>
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<td>Isoleucine</td>
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<td>Threonine</td>
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<td>Methionine</td>
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<td>Valine</td>
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<td><strong>Free fatty acids and related</strong></td>
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<td>acids and related metabolites</td>
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<td>Azelate</td>
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<td>Butyrate</td>
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<td>Carnitine</td>
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<td>Caprate</td>
<td>Fumarate</td>
<td>2-Oxoglutarate</td>
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<td>Caprylate</td>
<td>Succinate</td>
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<td><strong>Glycolysis</strong></td>
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<td>Pyruvate</td>
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<td><strong>TCA cycle intermediates</strong></td>
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<td>cis-Aconitate</td>
<td>Citrate</td>
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<td>Fumarate</td>
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<td></td>
<td>Succinate</td>
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<tr>
<td><strong>Others</strong></td>
<td>2-Aminobutyrate</td>
<td>Ascorbate</td>
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<td></td>
<td>Choline</td>
<td>Betaine</td>
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<td></td>
<td>Glycerophosphocholine</td>
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<td>Urea</td>
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Data from Smilowitz et al. [19] and Spevacek et al. [20].
which are located on chromosome 19 [19, 26]. Production of 2′FL or 3FL is highly correlated such that as the concentration of 2′FL increases, 3FL decreases (Pearson’s correlation $r = -0.76$; Fig. 1a). In women who are secretors (having a functional $FUT2$ gene), during the course of the first 3 months of lactation 2′FL decreases from approximately 8 mmol/L in colostrum to 3 mmol/L, whereas 3FL increases from approximately 0.3 mmol/L in colostrum to 1.6 mmol/L, where the concentrations of each oligosaccharide begin to level out [19, 20] (Fig. 1b). The reason for this correlated change in concentration is not fully understood. However, human milk oligosaccharides have been credited with developing the microbiota, and in particular selecting for and maintaining high levels of *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) in the infant GI tract during the period of exclusive human milk feeding, in addition to helping to build the immune system [9, 10]. These 2 oligosaccharides have also been recognized as having antiviral properties through acting as decoys to prevent binding of viruses to the GI tract [27, 28]. Human milk oligosaccharides have been reported to be absorbed into the blood, and 2′FL has specifically been shown to decrease plasma
cytokine levels [29]. Additionally, 2′FL has been shown to modulate CD14 expression on human enterocytes to attenuate LPS-induced inflammation [30]. Controlling systemic inflammation [25] may be particularly important in the first few months of life, as circulating levels of cytokines such as IL-6 may modulate fatty acid metabolism and induce insulin resistance [reviewed in 16], which could have important consequences on metabolic development.

Another interesting metabolite that increases over time in human milk is urea. We observed that it increases from 2.7 mmol/L in colostrum to 4.5 mmol/L on postpartum day 90 [19, 20]. This increase may be a way to help create a steady nitrogen source for the growing populations of microbes in the infant GI tract [19]. While most of the free amino acids are low in concentration in human milk throughout lactation (<1 mmol/L), several do increase over time (Table 1). In particular, glutamate increases from 0.5 mmol/L in colostrum to 1.5 mmol/L in mature milk over the first 3 months [19, 20]. Dietary glutamate is extensively metabolized by the intestinal epithelium [reviewed in 31], and this increase may be important for maturation of the intestinal tract of infants; however, more research needs to be done to fully understand the role of milk-derived free glutamate in the developing neonate.

*Human Milk and Infant Health*

The impact of human milk on infant metabolism can be demonstrated through comparison of the metabolomes of infants that have been formula fed and breastfed. Breastfed infants have been reported to have higher total cholesterol and LDL-C than formula-fed infants [32]. Additionally, breastfed infants have lower levels of short-chain unsaturated and higher levels of longer-chain polyunsaturated fatty acids containing phosphatidylcholines [33]. In contrast, breastfed infants have lower levels of polyunsaturated fatty acid-containing long-chain triglycerides, and higher levels of shorter-chain sphingomyelin and 16:0 and 20:4 cholesterol esters than formula-fed infants [33]. Breastfed infants also have higher fasting levels of acetate, acetone, myoinositol, glutamine, proline, and formate, as well as lower levels of urea, creatine, essential amino acids, and their by-products (threonine and valine, 2-hydroxybutyrate and 3-hydroxyisobutyrate), choline, and dimethyl sulfone than formula-fed infants (Table 2) [34]. In the postprandial state, breastfed infants have higher acetate, acetone, myoinositol, formate, methanol, and betaine, and lower 2-hydroxybutyrate, 3-hydroxyisobutyrate, alanine, isoleucine, leucine, lysine, methionine, proline, threonine, tyrosine, valine, choline, creatine, dimethyl sulfone, and urea (Table 3) [34]. Similar differences were observed in nonhuman primates [25].

Analysis of the urine metabolome of formula- and breastfed infants largely mirrors what is observed in the serum metabolome, with differences in metabo-
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The urine metabolome also reveals differences in gut microbial function, as was observed with lower TMAO (trimethylamine-N-oxide) in breastfed compared with formula-fed infants [25].

Analysis of the fecal metabolome can reveal functionality of the microbes within the GI tract of infants. For example, fecal metabolome analysis of breastfed infants revealed evidence of lower protein fermentation [36] and lower levels of short-chain fatty acids (propionate, butyrate, and acetate) and free amino acids, as well as higher levels of lactate and fucosylated oligosaccharides compared with formula-fed infants [35, 37, 38]. Measurement of fucosylated oligosaccharides in the stool is directly related to the consumption of human milk, as bovine milk (which the majority of formulas are based from) does not contain fucosylated oligosaccharides. Differences in lactate and short-chain fatty acids between breastfed and formula-fed infants can also be correlated with the resident microbes in the GI tract of breastfed infants. Compared with formula-fed infants, breastfed infants have higher levels bacteria from the Bifidobacterium and Lactobacillus genera, which produce lactate and acetate as primary fermentation products [10]. Acetate is higher in the plasma of breastfed infants (Tables 2, 3), which may suggest that acetate produced by these bacteria may be absorbed. Evidence of temporal changes in the fecal metabolome has also been reported [24], which implies important changes in microbial structure and function during development.

Table 2. Semifasted serum metabolite differences between breastfed and formula-fed infants

<table>
<thead>
<tr>
<th></th>
<th>Higher in breastfed</th>
<th>Lower in breastfed</th>
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<tbody>
<tr>
<td>Sugars</td>
<td>Myo-inositol</td>
<td></td>
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<tr>
<td>Ketone bodies</td>
<td>Acetone</td>
<td></td>
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<tr>
<td>Amino acids and related</td>
<td>Glutamine</td>
<td>Threonine</td>
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<tr>
<td>metabolites</td>
<td>Proline</td>
<td>Valine</td>
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<td></td>
<td></td>
<td>2-Hydroxybutyrate</td>
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<td></td>
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<td>3-Hydroxyisobutyrate</td>
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<tr>
<td>Urea cycle</td>
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<td>Urea</td>
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<tr>
<td>Short-chain fatty acid</td>
<td>Acetate</td>
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<tr>
<td>Others</td>
<td>Formate</td>
<td>Choline</td>
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<td></td>
<td></td>
<td>Creatine</td>
</tr>
<tr>
<td>Microbial metabolites</td>
<td>Methanol</td>
<td>Dimethyl sulfone</td>
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</tbody>
</table>

Data from Slupsky et al. [34].
Although there are common metabolome differences reflected in the blood, urine, and feces between breastfed and formula-fed infants, it is important to note that the composition of formulas vary depending on the study. Indeed, formulas on the market vary widely with differences in macro- and micronutrient composition. Changing one component in the formula can result in profound changes to an infant’s metabolism, the structure and function of the gut microbiome, or both. For instance, a recent study revealed that replacing lactose in infant formula with corn-syrup solids resulted in a lowering of many amino acids measured in the postprandial state (1–2 h) compared with infants fed lactose-based formula [34]. While this seems to be desirable, 2 h after feeding, glucose, ketones, and nonesterified fatty acids were lower in the infants fed formula with corn-syrup solids compared with those fed lactose-based formula, and their insulin levels were significantly higher than in breastfed infants.

Other studies have looked at adding probiotics to infant formula [24, 39, 40]. For example, supplementation of nonhuman primates with a formula containing *B. animalis* subsp. *lactis* resulted in slightly increased serum BCAA, and a structuring of the microbiota that was different from the formula-fed infants at

<table>
<thead>
<tr>
<th>Table 3. Postprandial serum metabolite differences between breastfed and formula-fed infants</th>
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Data from Slupsky et al. [34].
3 months [24]. Of potential concern was the marked increase in the fecal poly-
amines cadavarine and putrescine in the third month. Supplementation of hu-
mans infants with a formula containing *Bifidobacterium* *bifidum*, *B. breve*, *B. longum*, and *B. infantis* resulted in changes in both the structure and function (assessed through the fecal metabolome) of colonic bacteria during the period of supplementation but had no detectable long-term effects [39]. Another study where infant formula was supplemented with a probiotic/prebiotic combination of *B. animalis* subsp. *lactis* CNCM I-3446 and bovine milk oligosaccharides revealed an α diversity that was similar to breastfed infants over the course of sup-
plementation, in addition to higher levels of the *Bifidobacterium* genus in gen-
eral compared with control formula [40], but no metabolome changes were mea-
sured and reported. The fact that changes in metabolic parameters were observed in 2 of these studies suggests that these early gut microbes impact metabolic de-
velopment of the infant and further that a metabolic assessment should be con-
sidered an important outcome when evaluating changes in formula ingredients.

**The Collaboration between Milk and the Developing Neonate**

The components of human milk are unique and are specific to the growing neo-
nate. Replacing human milk with milk from other animals has profound conse-
quences for the developing neonate. For instance, the consistently observed in-
creases in plasma amino acids in formula-fed infants may inhibit several early steps in the insulin signaling cascade [41], and the sustained increases could contribute to hepatic mitochondrial dysfunction that may be associated with future increased BMI, insulin resistance, and dyslipidemia [42].

Components of human milk may also interact directly with specific meta-
bolic pathways, such as the mechanistic target of rapamycin (mTOR) pathway [43], to optimize development. mTOR complex 1 (mTORC1) is a nutrient-sen-
sitive kinase that plays an important role in many aspects of cell growth, protein and lipid synthesis, as well as lipid accumulation and adipogenesis. It is particu-
larly important in child development for the control of growth and metabolism of bone, skeletal muscle, the central nervous system, the GI tract, blood cells, and other organs [reviewed in 44]. Amino acids such as leucine can regulate mTORC1, and a correlation between the amount of leucine in the whey fraction and serum leucine levels in infants has been reported [45]. Bacteria, such as *Lactobacillus plantarum*, have also been shown to act on the TOR-dependent host nutrient-sensing system in *Drosophila* [46]. Interestingly, it was recently ob-
served that *L. plantarum* might be vertically transmitted from mother to infant through breastfeeding [47]. Thus, there may be a connection between the types of microbes colonizing the infant GI tract and expression of the mTOR pathway, although more studies are needed to confirm.
Conclusions

Diet is remarkable in both its ability to shape gut microbiota as well as metabolism and the immune system. The exquisite linkage of microbiota, host immunity, and host metabolism hints at the complexity of human milk, and how it has been optimized for neonatal development. The changes in human milk composition throughout lactation (increases and decreases in many metabolites) likely reflect the changing needs of the neonate and the gut microbiota. Analysis of the metabolome (either serum or urine or both) reflects the human phenotype and should be considered an essential component of any study that aims to capture the metabolic effects of diet. Analysis of the fecal metabolome can inform on the function of the gut microbes. Once a healthy baseline has been defined, the response to diet can be assessed through analysis of the serum, urine, and fecal metabolomes both in the short term and long term.

More studies on human milk should be done to assess how secretor status and Lewis blood type (both in the mother and the infant) affects infant development including immunity and metabolism, as well as other factors such as maternal health and environmental exposures. Incorporating additional data including genetic and epigenetic data will be important to understand individual responses to diet and microbial succession. Incorporating a nutrigenomic approach together with an analysis of microbial structure and function will also help us understand, on a deeper level, how human milk and its components affect gene expression either by themselves or through the gut microbiota. This approach will take many years but may ultimately allow us to understand the interplay between food, gut microbiota, and metabolism, and overall provide a better understanding on how to achieve optimal health through diet.

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Disclosure Statement

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Abstract
Human milk oligosaccharides (HMOs) are the third most abundant component of human milk. So far, more than 150 different and structurally distinct HMOs have been identified. HMO composition varies substantially between women, but remains fairly constant over the course of lactation in the same woman. Which maternal genetic and environmental factors drive the interindividual variations in HMO composition remains poorly understood, and it is currently unknown whether or not a woman’s characteristic HMO composition has evolved to match her own infant’s specific needs. A combination of preclinical, cohort, and clinical studies is required to fully assess the many effects, functions, and potential claims associated with HMOs. In some cases, individual HMOs exert a certain effect and, while there might be some redundancy, the effects are often highly structure-specific. In other cases, a combination of different HMOs in specific ratios to each other is required to be effective, and future research needs to assess whether or not the administration of individual HMOs alone may be counterproductive and potentially harmful to the infant’s short- and long-term health. Overall, the personalized complexity of HMOs cannot be mimicked in artificial infant formula and provides yet another powerful reason to protect, promote, and support breastfeeding.

What Are Human Milk Oligosaccharides?

One liter of mature human milk contains 5–15 g of complex carbohydrates that are collectively called human milk oligosaccharides (HMOs). Their high concentration makes HMOs the third most abundant solid component of mature
milk, only exceeded by the concentration of lactose and total lipids and often exceeding the concentration of total proteins [1]. In comparison, the concentration of oligosaccharides in bovine milk is 100- to 1,000-fold lower, and many of the bovine milk oligosaccharides are structurally distinct from and less complex than HMOs. While more than 150 different HMOs have been identified so far, their composition follows a basic blueprint that connects the five building blocks glucose (Glc), galactose (Gal), N-acetylgalactosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac) in specific linkages (Fig. 1a) [2]. All HMOs carry lactose (Galβ1–4Glc) at the reducing end. Lactose can be elongated in the C3 position of Gal with either lacto-N-biose (Galβ1–3GlcNAc, type 1 chain) or N-acetyllactosamine (Galβ1–4GlcNAc, type 2 chain) (Fig. 1b). This ostensibly minor difference in the linkage between Gal and GlcNAc might be important for HMO digestion and bioavailability. While the β1–3 linkage in lacto-N-biose (type 1) cannot be cleaved by the infant’s intestinal β-galactosidase (lactase), the β1–4-linkage in N-acetyllactosamine (type 2) is likely a substrate for the enzyme, suggesting that type 2 chain HMOs like lacto-N-neotetraose (LNnT) are partially digested in the infant’s proximal small intestine, do not reach the infant’s distal small intestine and colon intact, and are therefore not available for absorption or microbial utilization. In contrast, type 1 chain HMOs like lacto-N-tetraose (LNT) cannot be cleaved by lactase, resist degradation in the infant’s small intestine, reach the colon intact, and are available for absorption or microbial utilization. In addition to chain elongation at the C3 position of Gal, the disaccharides lacto-N-biose or N-acetyllactosamine can also be added at the C6 position of Gal, leading to chain branching (Fig. 1c). The repeated addition of disaccharides to the growing chain leads to more complex linear or branched HMOs (Fig. 1d). Moreover, HMOs can be modified by the addition of Fuc in α1–2, α1–3, and/or α1–4 linkage and/or the addition of Neu5Ac in α2–3 and/or α2–6 linkage. Each Neu5Ac contains a carboxyl group and introduces a negative charge to an HMO. Fuc or Neu5Ac can be added to lactose to yield the small HMO trisaccharides 2′-fucosyllactose (2′FL), 3-fucosyllactose (3FL), 3′-sialyllactose, or 6′-sialyllactose (Fig. 1e). One or more Fuc and/or Neu5Ac can also be added to the growing linear or branched HMO chain to form complex HMOs with potentially multiple negative charges if more than one Neu5Ac are added (Fig. 1f). Neu5Ac-containing HMOs are often referred to as “acidic” HMOs.

Although HMO composition follows a basic blueprint and more than 150 different HMOs have been identified so far, it is important to note that every woman synthesizes and secretes a distinct HMO composition profile that varies substantially between different women, but remains fairly constant over the course of lactation for the same woman.
Fig. 1. Human milk oligosaccharide (HMO) blueprint and structural diversity. HMOs consist of the five monosaccharide building blocks glucose (black circles), galactose (Gal, grey circles), N-acetylglucosamine (squares), fucose (Fuc, triangles), and N-acetyleneuraminic acid (NeuAc, diamonds). a The HMO structural composition follows a basic blueprint. b Lactose forms the reducing end and can be elongated in the C3 or C6 position of Gal with either lacto-N-biose to form type 1 chains or with N-acetyllactosamine to form type 2 chains. While the β1–3 linkage in lacto-N-biose (type 1) cannot be cleaved by the infant's intestinal β-galactosidase (lactase), the β1–4 linkage in N-acetyllactosamine (type 2) is likely a substrate for the enzyme (indicated by scissors), suggesting that type 2 chain HMOs like lacto-N-neo-tetraose are partially digested in the infant's small intestine and are thus not available in their intact form for absorption or microbial utilization. c Addition of either of the two disaccharides in β1–6 linkage leads to HMO branching. d The repeated addition of disaccharides to the growing chain leads to more complex linear or branched HMOs. e, f Lactose (e) or the elongated or branched chains (f) can be modified with Fuc in α1–2, α1–3, or α1–4 linkage to yield fucosylated HMOs. Lactose or the elongated or branched chains can also be modified with NeuAc in α2–3 or α2–6 linkage to yield sialylated HMOs, occasionally also referred to as “acidic HMOs.” Some examples of the diverse collection of HMOs are provided.
Some of the variation in HMO composition can be explained by maternal genetics [1]. HMO fucosylation is mainly catalyzed by two different fucosyltransferases, enzymes that facilitate the addition of Fuc to the HMO backbone in different linkages [3, 4]. Fucosyltransferase 2 (FUT2) adds Fuc to Gal in an α1–2 linkage [5]. Mutations in the Secretor gene, which encodes FUT2, lead to an inactivation of the enzyme, and, as a consequence, milk samples of so-called nonsecretor women have very low concentrations of α1–2-fucosylated HMOs, e.g. 2′-FL or lacto-N-fucopentaose 1 (LNFP1). Fucosyltransferase 3 (FUT3) adds Fuc to Gal or GlcNAc in an α1–3 or α1–4 linkage, depending on whether the substrate is a type 1 or type 2 HMO chain [6]. Mutations in the Lewis gene, which encodes FUT3, lead to an inactivation of the enzyme, and, as a consequence, milk samples of so-called Lewis-negative women have very low concentrations of α1–3/4-fucosylated HMOs. The permutation of active/inactive FUT2 and FUT3 enzymes leads to four distinct HMO groups [4]: (a) Lewis-positive secretors (FUT2 and FUT 3 active), (b) Lewis-positive nonsecretors (FUT2 inactive, but FUT3 active), (c) Lewis-negative secretors (FUT2 active, but FUT3 inactive), and (d) Lewis-negative nonsecretors (both FUT2 and FUT3 inactive). These four groups can be distinguished by the presence or near absence of specific fucosylated HMOs, which is almost an all-or-nothing phenomenon. For example, 2′FL is either present and one of the dominant HMOs in the milk of secretor women (active FUT2), or it is almost completely absent in the milk of nonsecretor women (inactive FUT2). In contrast, the variation of HMOs that do not depend on FUT2 or FUT3 activity is subtler. In fact, it remains poorly understood which enzymes other than FUT2 and FUT3 are involved in HMO biosynthesis in the human mammary gland epithelial cell.

In addition to maternal genetics and potentially epigenetics, maternal environmental factors may also affect HMO composition. Our recent work with mice has shown that milk oligosaccharide concentrations decrease when lactating dams are fed a high-fat diet and increase when dams are exercising [unpubl. data]. How diet, exercise, and other lifestyle factors impact HMO composition in humans is currently under investigation. Maternal health may also affect HMO composition. Initial preliminary data suggest that obesity or chronic inflammatory diseases impact HMO composition [unpubl. data]. In addition, cross-sectional data from the Canadian Healthy Infant Longitudinal Development (CHILD) national birth cohort [7] indicate that parity increases overall HMO concentration, but maternal age, method of delivery, or infant gender showed no association with HMO composition [unpubl. data].
What Happens to HMOs after Ingestion?

Once ingested, HMOs resist the low stomach pH as well as degradation through pancreatic and brush border enzymes in the small intestine [8, 9], with the potential exception of type 2 chains in which the terminal β1–4-linked Gal may be cleaved off by the enzyme lactase. Approximately 1% of the ingested HMOs are absorbed and can be measured in the systemic circulation as well as in the urine [10–13], indicating that HMO effects extend to tissues and organs other than the intestine. Most HMOs reach the distal small intestine and colon intact where they are either metabolized by microbes or excreted with the feces.

What Are Potential HMO Functions?

HMOs serve as metabolic substrates for specific and potentially health-promoting bacteria in the infant’s intestine [14–16], making them the first prebiotics that humans are exposed to in life – when the infant is breastfed (A, Fig. 2). However, recent data from ex vivo studies suggest that the prebiotic effects of different HMOs are not interchangeable and are indeed structure-specific [unpubl. data]. The composition of microbial communities isolated from infant fecal samples and cultured under anaerobic conditions changes over time depending on what specific HMOs are added to the culture. For example, the composition of a microbial community looks very different when exposed to either a mixture of HMOs that were isolated from pooled human milk or to individual HMOs like 2′FL or LNT. Since the differential composition and activity of microbial communities has been linked to diseases like obesity, diabetes, inflammatory bowel disease or autism, exposing infants to individual HMOs in formula instead of a complex HMO mixture in human milk may increase disease risks. Long-term follow-up studies are required to rule out this potential risk and avoid potential harm to the infant’s short- and long-term health.

However, HMOs are more than just “food for bugs.” HMOs can have direct bacteriostatic or bacteriocidal antimicrobial effects (B, Fig. 2). For example, HMOs halt the growth of Streptococcus agalactiae (group B Streptococcus; GBS) [17], a leading cause of invasive bacterial infections in newborns, typically acquired vertically during childbirth secondary to maternal vaginal colonization. GBS transmission to the newborn is associated with risk of pneumonia, septicemia, and meningitis [18–20]. Multidimensional chromatography revealed that the bacteriostatic effect is structure dependent and that LNT is most effective. GBS transposon mutant library screening identified a GBS glycosyltransferase as the HMO target. Most intriguingly, the effects of HMOs synergize with com-
mon antibiotics like vancomycin and ciprofloxacin, with high relevance to the emerging antibiotic resistance crisis [17].

HMOs also have antiadhesive effects (C, Fig. 2). Many pathogens need to attach to epithelial surfaces in order to proliferate and potentially invade host tissues. The attachment is often facilitated by pathogen surface molecules that bind to glycan structures on the epithelial cell surface, also known as the glyco- calyx. HMOs and glycans on epithelial cell surfaces share many structural features, allowing HMOs to mimic epithelial surface glycans and serve as soluble decoy receptors. Instead of pathogens binding to epithelial surfaces and causing disease, they bind to the soluble HMO decoys and are washed out without attaching and without causing disease. Examples for antiadhesive effects of HMOs include bacterial pathogens like Campylobacter jejuni [21] or enteropathogenic Escherichia coli [22] as well as protozoan parasites like Entamoeba histolytica [23].
HMOs may also have direct effects on epithelial cells, independent of, but indirectly affecting microbes (D, Fig. 2). For example, bladder epithelial cells that had been exposed to HMOs are significantly more resistant to uropathogenic *E. coli* invasion and cytotoxicity [24]. HMOs may also alter immune cell responses, either locally in the gut or systemically (E, Fig. 2).

In conclusion, there are indirect effects of HMOs on the infant that are mediated through changes in microbial communities, e.g. by serving as prebiotics, antimicrobials, or antiadhesives, and there are direct effects on the infant, e.g. by modulating epithelial or immune cell responses. In addition, there may be multiple other mechanisms of HMO action that have not yet been discovered. Overall, the arsenal of HMO effects is likely relevant in a variety of health and disease contexts.

**How Do We Interrogate the Effects of HMOs?**

Accumulating data from tissue culture, animal, or human cohort studies suggest that HMOs benefit the human milk-fed infant. However, results from many of these studies raise additional questions. How relevant are results generated from in vitro or animal models? How similar are cell lines in culture compared to tissues in the infant body? How comparable are animal models to human anatomy, physiology, and pathophysiology? How meaningful are associations between HMOs and infant outcome measures in human cohort studies? Are the observed associations true cause-and-effect relationships? No one single study alone will likely provide a comprehensive answer. Instead, a thoroughly designed interrogative strategy that combines preclinical studies in tissue culture and animal models with clinical cohort analyses and eventually clinical intervention studies will be required to fully assess the many effects, functions, and potentials claims associated with HMOs. Our recent work on necrotizing enterocolitis (NEC) serves as one example of how the combination of preclinical studies and human cohort studies can inform clinical intervention studies to test the hypothesis that HMOs contribute to the beneficial effect of human milk feeding.

NEC is one of the most common and fatal gastrointestinal disorders in preterm infants [25]. About 5% of all very-low-birthweight (VLBW) infants develop NEC, with a mortality rate of over 25%. While formula-fed infants are at a 6- to 10-fold higher risk to develop NEC [26], the protective mechanisms of feeding human milk remain poorly understood. In contrast to formula, human milk is an abundant source of HMOs. In addition, the NEC-preventing effect of human donor milk persists even after pasteurization, a process that destroys and inactivates many human milk bioactives, but keeps HMOs intact and active.
Based on these observations, we hypothesized that HMOs contribute to the lower NEC incidence in human milk-fed infants, and tested the hypothesis in a neonatal rat model [27]. Newborn rat pups were either left with the dam to serve as "breastfed" control or removed from the dam and received formula by oral gavage. While all of the dam-fed pups survived the first 4 days of life, only ~75% of the formula-fed pups survived. However, 95% of the pups survived when they received a formula that was supplemented with HMOs that were isolated from pooled human milk. This significant increase in survival coincided with decreased pathological observations, both macroscopically as well as microscopically. We then applied a multidimensional chromatography approach to separate the different HMOs first by charge and later by size, and identified one specific HMO, disialyllacto-N-tetraose (DSLNT), which contains two Neu5Ac, to be responsible for the beneficial effects. Enzymatic removal of just one of the Neu5Ac led to a complete loss of function, indicating that the effect is highly structure-specific [27].

While the data were encouraging, the validity of available preclinical NEC models in rodents or piglets is limited [28]. Animals are exposed to external hypoxic and/or hypothermic insults that are rather artificial, and the use of animals itself is a limitation due to interspecies differences in gastrointestinal development, anatomy, and physiology. Thus, advancing a potential therapeutic like DSLNT from controversial preclinical models to clinical treatment trials carried a tremendous risk of failure. To help close the gap between animal models and clinical intervention studies, we used an intermediate approach and conducted a multicenter clinical prospective cohort study with mothers and their VLBW infants fed predominantly human milk [29]. The study was based on the observation that some infants still develop NEC despite receiving predominantly human milk. Since HMO composition varies between women, it led us to hypothesize that infants who develop NEC received milk with less DSLNT than infants who do not develop NEC. We recruited 200 mothers and their VLBW infants that were predominantly human milk-fed. We analyzed HMO composition in human milk fed to infants over the first 28 days postpartum, matched each NEC case with 5 controls, and used logistic regression and generalized estimating equation to show that DSLNT concentrations were significantly lower in almost all milk samples in all 8 NEC cases when compared to controls. In fact, DSLNT abundance could identify NEC cases prior to onset. Aggregate assessment of DSLNT over multiple days enhanced the separation of NEC cases and control subjects, making DSLNT content in human milk a potential noninvasive marker to identify infants at risk of developing NEC. While the cohort association data alone would raise questions about cause-and-effect, the combination with data from the preclinical model substantially increases the confidence that the
observed effects are indeed due to DSLNT, lowering the risk threshold for a clinical intervention study to fail.

Overall, the NEC project is an example of how the combination of preclinical data and clinical cohort data can inform clinical intervention studies (Fig. 3). A similar approach has been used in the past to relate HMOs to a reduction of *C. jejuni* infection and diarrhea [21, 30] and is currently used to study the effects of HMOs on infant body composition and childhood obesity risk as well as on allergies and asthma risk.
What we have learnt so far from this combined approach is that sometimes specific HMOs are effective and those effects are usually highly structure-specific and dose-dependent. The underlying mechanisms are likely mediated by specific receptors on host (infant) tissues or on microbes. In other cases, a combination of different HMOs in specific ratios to each other is required to be effective. The underlying mechanisms are likely mediated indirectly through shaping microbial communities or directly through a coordinated interaction of different HMOs with multiple different receptors or even different cell types, for example in the immune system. In situations where relative abundances of many different HMOs matter more than individual HMOs alone, it raises the question whether or not a woman’s characteristic HMO composition has evolved to match her own infant’s specific needs. In that case, HMOs can be seen as another example of personalized nutrition early on in life, which comes in addition to other personalized components of human milk like antibodies, milk microbiota, immune cells, and progenitor cells. In fact, the personalized complexity of HMOs provides yet another powerful reason to protect, promote, and support breastfeeding.

Disclosure Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Research Gap and Opportunities


Abstract

Significantly preterm and low-birthweight (LBW) babies have diminished lung and gut development, generally fail to thrive, have increased mortality and higher frequency of mature-onset disease. Mothers often cannot breastfeed, and babies receive either formula or pasteurized donor milk, which may further limit the baby’s recovery. New approaches are required to manage the early stages of neonatal development. The tammar wallaby, an Australian marsupial, has a short gestation and a simple placenta, and gives birth to an altricial young equivalent to a final trimester human embryo. The neonate remains in the pouch and attached to the teat for 100 days postpartum. The mother slows growth of the young and progressively changes the composition of the milk to deliver signals for organ development, including the lung and gut. This closely resembles the relationship between the human fetus and delivery of placental and uterine bioactives. Datasets comprised of differentially expressed genes coding for secreted proteins in early lactation in the tammar mammary gland have been compared to databases produced from human placenta, amniotic fluid, colostrum and milk to identify human homologues for the putative signaling molecules for organ development. These data will be used to develop milk fortifiers for treatment of preterm and LBW babies in both the developed and the developing world.

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**Introduction**

Significantly preterm and low-birthweight (LBW) babies have acute challenges for survival, largely due to limited development of their lungs and gut. Furthermore, disruption to the timing of developmental programming in the neonate often results in increased frequency of mature-onset disease, and this is exacerbated if growth rates are accelerated too aggressively [1]. The cost to manage these babies in hospitals is considerable, and there is an increased prevalence of this problem in the developing world. Mothers often cannot breastfeed, and the only option available is providing either formula or pasteurized donor milk to improve the development of these babies. New approaches are required to manage the early stages of treatment, and particularly a focus on the development of organs without an accompanying large increment in growth.

Milk has multiple functions. Evolutionary pressure on mammals has ensured that milk provides the most appropriate nutrition for growth of the newborn. It also has the capacity to remodel the mammary gland and can provide protection from infection and inflammation when the mammary gland is susceptible to these challenges. Innovative studies are now exploiting the unusual reproductive strategy of an Australian marsupial, the tammar wallaby (*Macropus eugenii*) to identify milk bioactives that impact on tissue development in the neonate [2, 3]. Reproduction in the tammar wallaby is characterized by a short 26-day gestation, a simple placenta [4], birth of an immature young, and a relatively long 300-day lactation (Fig. 1a, b). During the first 100 days postpartum, the development of the neonate is similar to that of a eutherian fetus in its final trimester [2, 3].

**Fig. 1.** a The tammar as a model system for premature and low-birthweight babies. The tammar young is 6 days of age, the human neonate is 26 weeks of age. b Tammar wallaby lactation strategy. Progressive changes in milk composition and growth of the young during the three phases of the lactation cycle in the tammar wallaby. Total protein concentration does not change significantly during early lactation but there is a progressive change in the kinds of proteins secreted. c Differential expression of the major milk proteins during tammar lactation. The profile of α-casein (α-CAS), β-casein (β-CAS), α-lactalbumin (α-LAC), β-lactoglobulin (β-LG), early lactation protein (ELP), whey acidic protein (WAP), late lactation protein-A (LLP-A), and late lactation protein-B (LLP-B) gene expression. d Fostered pouch young. Pouch young at day 120 of age were either cross-fostered to host mothers at day 170 of lactation or retained on mothers at day 120 of lactation. After 50 days, both pouch young were removed. The more mature animal (shown on the right) was fostered to a mother at a more advanced stage of lactation. Adapted from Sharp et al. [3].

*(For figure 1 see next page.)*
Both the tammar newborn and the human preterm baby will receive milk to sustain their development. The preterm baby will continue to face serious acute and chronic health challenges that may limit a timely recovery. In contrast, the tammar neonate will receive milk that is appropriate for growth and development of a healthy young. Therefore, can an understanding of the milk bioactivity provided to the tammar neonate underpin new approaches to develop a human milk fortifier to better manage preterm babies?

During early lactation in the tammar, the immature organs that are necessary for their survival such as respiratory system, gut, lymphoid tissues, and nervous system, including brain and spinal cord, are rapidly developed [5–9]. The major and minor milk constituents change substantially and progressively during lactation in the tammar, and these changes have been shown to regulate growth and development of the tammar pouch young, allowing specific changes in milk composition to be correlated with specific developmental events [2, 3]. Interestingly, during the first 100 days of lactation, the mother delivers milk with a composition that slows growth of the young but augments an increased rate of organ development [10]. Transferring young to a mother at a more advanced stage of lactation accelerates this process and supports the argument that exposure of the young to a more mature milk with a range of new bioactivity has the potential to impact on development of the fostered young (Fig. 1d) [6, 11, 12]. Therefore, the signaling factors involved in the development of the eutherian fetus, most of which would be derived from the placenta, amniotic fluid, and perhaps colostrum, are most likely delivered in the milk of marsupials [6, 11, 12].

Since the appearance of the aplacental, egg-laying monotremes 200 million years ago, there has been extensive adaptation to reproduction, particularly in lactational strategies when the Theria split into the Metatheria (Marsupialia) and Eutheria (Placentalia) lineages over 140 million years ago [13]. In contrast to marsupials, eutherians have a well-developed placenta and a long gestation that leads to the birth of a relatively well-developed young. The length of lactation is often similar to gestation, and composition of the milk does not change substantially. This is consistent with the concept that signaling molecules are presented by the placenta and amnion prior to parturition. Therefore, a strong argument is growing for a comparative approach to better identify the specific signals that regulate development. The regulatory mechanisms controlling the great majority of physiological processes have been conserved during evolution, but the timing and mechanism for delivering these processes may differ between species of mammals [2, 3]. Therefore, the use of these diverse species, coupled with the availability of current technologies, provides the opportunity to exploit marsupial models for new insights into the functions of milk. This may lead to
a new range of human fortifiers that include bioactives with the potential to specifically target the growth and development of tissues in the human neonate to improve outcomes for premature and LBW babies.

**Regulation of the Tammar Lactation Cycle**

The tammar lactation cycle is divided into four broad phases (Fig. 1b) [2, 3, 10]. Phase 1 is comprised of a 26-day gestation including the subsequent birth of the altricial young which climbs into the pouch and attaches to one of four teats. During the first 100 days postpartum (phase 2A), the immature neonate remains permanently attached to the teat and has limited capacity to mount an immune response [7]. The mother produces relatively small volumes of dilute milk containing a high concentration of complex carbohydrates and a low concentration of protein and lipid (Fig. 1b). The conversion of milk to body mass during this phase is similar to that reported for a eutherian fetus [10]. Phase 2B commences 100 days postpartum and continues for approximately 100 days during which the neonate remains in the pouch, relinquishes the teat, and continues to suckle intermittently. The milk produced is maintained with high levels of carbohydrates and low concentrations of protein and lipids until the onset of phase 3 when the young begins to exit the pouch and feeds on herbage, but continues to suckle. During phase 3, the mammary gland enlarges significantly, producing large amounts of milk that is rich in protein and lipid but low in carbohydrates to provide a high-energy milk. Recent experiments have shown that the lactation program, and particularly these changes in milk composition are regulated by the mammary extracellular matrix [14].

During tammar lactation, casein, α-lactalbumin, and β-lactoglobulin genes are induced at parturition and remain expressed throughout lactation which is similar to the eutherian mammals. However, there are significant temporal changes in expression of some of the major milk protein genes and many of the minor milk protein genes (Fig. 1c) [2]. In addition, microarray analysis of the tammar mammary gland has revealed a multitude of changes in gene expression during the lactation cycle [2]. The tammar-specific microarray was generated from a normalized mammary gland cDNA library produced using an expression vector. Therefore, specific proteins could be synthesized in vitro following transfection of the plasmid into CHO cells [2] and secretion of the protein into the culture media. This process produced enough protein to examine bioactivity in any relevant in vitro model and subsequently revealed temporal delivery of a range of bioactivities. The microarray analysis also confirmed expression of
some major milk proteins expressed in each phase of lactation that were used as markers to examine the regulation of the lactation program in the mammary gland [2, 14].

**The Role of Milk Bioactives in Development of Specific Tissues in the Suckled Tammar Neonate**

*Development of the Gut*

Previous studies have shown dramatic changes in gut morphology occur in the suckled young and take place while the young is still in the pouch [6, 15]. In the hindstomach region, parietal cells increase in number, gastric glands enlarge and adopt the adult-like phenotype, and peptic enzyme activity becomes elevated. Concomitantly, the forestomach region changes from an immature gastric glandular phenotype to a cardia glandular phenotype in the region that becomes the adult forestomach. These changes in stomach morphology were correlated with significant changes in milk composition, and a study that transferred pouch young to host mothers at a more advanced stage of lactation for 50 days indicated that the process of forestomach maturation was accelerated [6].

More recent studies have shown that milk collected from tammars in the first 100 days of lactation and cultured with stomach explants from day 12 mouse embryos resulted in elevated cell proliferation and increased level of expression of specific developmental gene markers [Kurappath et al., unpubl.]. Therefore, identification of these developmental signaling molecules in tammar milk will show promise for new strategies to address limited gut development.

*Development of the Lung*

In eutherians, the majority of lung morphogenesis occurs during gestation to enable gaseous exchange at birth. In contrast, studies of lung development in several marsupial species, including the tammar wallaby [3] have demonstrated that the major developmental changes in the respiratory system occur during early postnatal life [5]. A recent study using *Monodelphis domestica* (the American opossum) has shown that major postnatal development of the lung was similar to the tammar neonate. RNAseq analysis of the *Monodelphis* lung during each major stage of development was compared with RNAseq analysis of the mouse lung in embryos and suggested that the potential regulatory processes were very similar despite the timing of this development in the neonate and fetus, respectively [Modepalli et al., unpubl.]. Therefore, it is likely the lung is signaled by a conserved mechanism provided by either the milk in marsupials or the uterine environment in eutherians.
The potential role of tammar milk in lung development was examined using mouse embryonic lungs (E-12) cultured in media with tammar skim milk collected at key time points during lactation (Fig. 2a) [16]. The embryonic lungs showed increased branching morphogenesis when incubated with milk collected between day 40 and 100 of lactation, and reduced lung development when incubated in media with milk from day 20 of lactation (Fig. 2b–d) [16]. In addition, day 60 milk significantly upregulated a number of marker genes for key developmental processes and specialized cell types including the potential to increase production of surfactant (Fig. 2f) [16]. This temporal effect was lost in milk collected from day 100 to 200 of lactation.

The mechanisms by which the day 60 milk stimulated lung development were examined further [16] to show that lung epithelial cells cultured on Matrigel in media with day 60 milk proteins had increased proliferation and formed organoid structures with a lumen. In separate experiments, the lung mesenchymal cells cultured on Matrigel showed increased proliferation and invaded the surrounding matrix with epithelial branching morphogenesis [16]. The mesenchymal cells became flattened, elongated, and spindle shaped, with a similar morphology to either airway smooth muscle cells or myofibroblast cells.

Cross-fostering experiments have been used to assess effects of milk composition on lung development in the tammar [17]. The pouch young at day 25 of age were fostered to a series of mothers at day 15 of lactation, so that the young only received milk from day 15 to 25 of lactation for a period of 20 days. Analysis showed that expression of marker genes related to branching morphogenesis, alveolization, and presence of terminal and airway epithelia were significantly reduced in lungs from fostered pouch young compared to lungs from control pouch young. These data are consistent with a diminished level of differentiation of the lung in fostered young.

Fig. 2. Effect of early-phase tammar wallaby milk on lung branching morphogenesis. a Lungs were removed from embryonic mice and cultured with 10% tammar milk. b–e Lungs showed extensive branching morphogenesis and increased volume after 3 days of culture with milk collected at day (D) 60 of lactation but not lungs cultured in media with either 10% PBS (control) or day 120 milk. Sections of lung stained with HE confirmed that the morphology of embryonic lungs treated with day 60 milk showed increased branching. f Effect of early-phase tammar wallaby milk on lung developmental gene markers. Marker genes for lung development were all significantly upregulated in lung cultured with day 60 milk. Expression of Sp-B and Sp-C genes indicated increased type II cells producing surfactant, wnt-7b and BMP4 gene expression increased confirming branching morphogenesis, and increased Id-2 gene expression confirmed cell proliferation and growth of the lung. Adapted from Sharp et al. [3]. * p < 0.005.

(For figure 2 see next page.)
Taken collectively, the analysis of lung development identified a window of bioactivity in milk samples collected between day 20 and day 100 of lactation. Subsequent analysis of proteins in milk at day 20, day 60, and day 120 of lactation using mass spectrometry identified 19 proteins, predominately growth factors that have the potential to directly stimulate lung development [17].

**Mechanisms for Delivery of Bioactivity in Milk**

The major bioactives in milk fall within a number of groups: proteins, peptides, complex carbohydrates, and miRNA. The proteins and peptides are of particular interest given that bioinformatics analysis of the tammar mammary transcriptome has revealed more than 100 secreted proteases and an almost equal number of secreted protease inhibitors [Watt et al., unpubl.]. This provides a very complex set of interactions and the potential for a huge repertoire of proteins and peptides that can be delivered temporally in the milk. In addition, there are examples of alternative splicing of mammary genes to deliver domain-specific proteins in a timed way to impact potentially on the mammary gland and the development of the young as required [2].

The tammar has also provided an interesting model to better assess the potential of milk miRNA delivered in exosomes to signal events in the young. The kinds of miRNA in tammar milk change during lactation, and there is evidence that some of the miRNA appear to be produced in the mammary gland, and can be transferred across the gut to the peripheral circulation in the suckled young. Therefore, it is likely milk miRNA represent not only potential markers of mammary gland development and activity during the lactation cycle, but also new putative signaling molecules involved in programming development of the suckled young [18].

**Human Milk Bioactivity: Acute Response to Challenge and Potential for Breast-Programmed Development**

The capacity of human colostrum and milk to signal development of tissue has not been extensively explored. However, breast milk does have the capacity to respond rapidly to short-term changes to breastfeeding without the need for any metabolic or genomic intervention. Recent experiments examined skim milk prepared from samples collected from women in mid-lactation and incubated at 37°C over a period of 7 days to allow digestion of milk protein by en-
Endogenous proteases [Watt et al., unpubl.]. Results showed peptides were predominantly derived from caseins with limited digestion of the whey proteins. Subsequent antibacterial assays using *Staphylococcus aureus*, a major cause of breast infection showed the milk peptides significantly increased antimicrobial activity. The peptides also showed increased levels of anti-inflammatory activity [Watt et al., unpubl.]. Importantly, the peptides did not show any capacity to program apoptotic activity in human mammary epithelial cells. Therefore, the milk has an immediate capacity to produce peptides that play a specific role in the breast to reduce infection and inflammation if there is an interruption to breastfeeding.

A more extensive response is observed during mastitic challenge to the breast and can be assessed by analyzing cells in milk. A recent study [19] showed microarray analysis of genes expressed in cells present in breast milk at days 24, 48, and 101 of lactation, and days 7 and 14 of involution provided a reasonable snapshot of the secretory activity of the breast, despite some changes in the types of cells in milk. Analysis of cells in milk from women with mastitis identified increased expression of a new class of genes not expressed in lactation. Subsequently, treatment of human mammospheres in culture with bacterial LPS showed similar genes were expressed, confirming a response is evident after directly challenging the mammary epithelial cells [Watt et al., unpubl.]. However, this kind of acute response is necessary to protect the breast and to maintain lactation and is not aligned with the programmed delivery of bioactives that stimulates tissue development observed in marsupials. The prospect of using genome expression databases generated from the tammar mammary gland during development of the neonate may prove useful to interrogate datasets derived from human colostrum, milk, placenta, and amniotic fluid to better understand the signaling of development in the human fetus and neonate.

The most significant change in the composition of human milk that equates with changes seen in the transition between phases of lactation in marsupials is during the transition from colostrum to milk. Colostrum has been identified as a rich source of growth factors, immunoglobulins, and other proteins that are important to provide a “positive start” to development of the newborn baby. However, it is timely to reexamine the potential role of colostrum for its impact on the development of the baby, and particularly to explore any influence of colostrum on either initiating or augmenting the development of specific tissues. Therefore, it is particularly relevant to query databases from these sources in the comparison with genes exclusively and differentially expressed in the tammar mammary gland during early lactation.
A Comparison of Tammar and Human Databases to Identify Human Colostrum, Milk, Placental, and Amniotic Fluid Bioactives

To better understand the concept that expression of putative signaling molecules in phase 2A milk from the tammar may inform us about the role of the placenta, amniotic fluid, colostrum, and milk in the development of neonatal tissues in the human, a number of genomic databases have been compared. The mammary glands from a total of 20 wallabies at phase 2A, 2B, and 3 of lactation were analyzed using RNAseq and microarray [2], and genes expressed either exclusively in phase 2A or with expression levels greater than 2-fold ($p < 0.05$) higher than genes in phase 2B and 3 were identified. Cells from human milk collected either at day 30 of lactation or from colostrum were analyzed by either RNAseq [Geddes and Twigger, unpubl. data] or microarray [19]. The genes expressed either exclusively or at levels 2-fold greater ($p < 0.05$) in cells from colostrum or milk were identified. Genomic datasets for human placenta and amniotic fluid were publically available and included in the study [1, 20]. Importantly, the only genes analyzed in this study were identified as coding for secreted proteins which potentially are made available to targets [2].

The first surprising observation was the identification of 105 genes common to the wallaby phase 2A mammary tissue and human colostrum. These genes are currently being analyzed further but indicate the potential of colostrum for having an impact on early development of the neonate, in addition to uterine-derived signalling. Additional datasets produced from the colostrum associated with significantly preterm and LBW babies will be essential to determine whether the same proteins are made available to term, preterm, and LBW babies. These data will be important not only to better understand the potential acute impact on the neonate but conceivably could be important for influencing the incidence of mature-onset disease that presumably is a consequence of the lack of signalling these events at a critical time point in the development of the baby. The comparisons of the tammar mammary gland and human placenta, and tammar mammary gland and the amniotic fluid identified additional genes present exclusively in the human dataset. These data show promise for identifying genes with the potential to regulate development of the human fetus and neonate and that may have been co-opted from the marsupial mammary gland.

Conclusion

The mammary gland in marsupial species is extremely sophisticated in terms of the molecular organization for temporal delivery of bioactives to multiple targets. Clearly, the role of the mammary extracellular matrix has evolved in euthe-
rians and has retained the function to regulate morphology and differentiation of the mammary gland but no longer has a role in changing the composition of the milk. It is now emerging that the marsupial provides a unique opportunity to more easily identify the bioactives that potentially play a role in early development of the fetus and neonate.

These outcomes are important for development of better therapeutic options to treat the limited lung and gut development in premature and LBW babies that fail to thrive and that can have a significantly higher rate of mortality. This opportunity may extend to investigating the significant programming of developmental clocks that occurs in the earlier stages of development and that subsequently impacts on the increased prevalence of mature-onset disease seen in these babies [21]. It will be interesting to determine whether the developmental program is set in marsupials during the characteristically short gestation, which can be as little as 8 days [10], or whether the milk is providing signals postnatally to the altricial neonate. These models also provide the option of cross-fostering neonates to mothers at other stages of lactation to exclude windows of potential delivery of putative milk bioactives at critical times in the developmental process and to examine the impact of increased growth rates which must be managed to ensure a better outcome for acute and chronic treatment of premature and LBW babies.

Disclosure Statement

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References

Research Gap and Opportunities


Milk Lipids: A Complex Nutrient Delivery System

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Abstract

The evolution of lactation and the composition, structures, and functions of milk’s biopolymers illustrates that the Darwinian pressure on lactation selected for gene products with considerable structural complexity and diverse functions within the digestive system. For example, complex sugar polymers – oligosaccharides – possess unique properties in guiding the growth of intestinal bacteria that are not possible by feeding their simple sugars. The proteins of milk are diverse with some exhibiting enzymatic activities towards other milk components rendering those components both more digestible but also releasing biologically active products. Thus, research into milk’s biopolymers has been most enlightening when milk was investigated for the formation and disassembly of its structures and for the functions within the infant. To date however, the most complex structure in mammalian milk, the fat globule, has not been effectively examined beyond its simple composition. The globules of milk are heterogeneous in size, composition, and function. With new research tools, scientists are beginning to understand the mechanisms that control the assembly of globules in the mammary gland and the disassembly within the infant.

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Introduction

Milk fat has been largely ignored by diet and health research apart from linking fat to levels of cholesterol in blood. As a result, milk fat has been viewed as a net damaging contribution to human diets and even for infants. Such a negative value is not justified by more broadly based biological perspectives. Computational analysis of the evolution of the repertoire of mammalian genes responsible for lactation produced an unexpected conclusion. The most conserved sequence identities within the lactation subset of genes through mammalian evolution are the genes responsible for lipid secretion [1]. From a purely evolutionary conservation perspective, such results position milk as a lipid delivery system. Nonetheless, it is not clear from disease protection or long-term performance what lipids are providing to infants. Similarly, it is not evident when genetic, environmental or nutritional factors in maternal mammary gland compromise the synthesis of milk lipids. This lack of mechanistic understanding of milk synthesis and globule assembly is an important barrier to policy for dietary intakes for mothers. Without a mechanistic understanding of functions of milk lipids, it is not possible to develop methods that are capable of diagnosing functional impairments in infants due to insufficiencies or excesses in particular fatty acids, complex lipids, or even metabolic cofactors. The diversity of lipid molecules in milk implies a diversity of functions, yet scientists are still focusing almost exclusively on fatty acids. Lipids are far more complex, and in that complexity resides the assembly of cellular membranes, components of dispersed particles, epithelial barriers in skin, surfactant lipids in lungs and vesicles within and without cellular compartments. In addition to diverse structural roles, lipids provide an array of substrates for metabolic fuel, and they provide a precursor pool for a range of signaling molecules. Recent developments in biochemistry, gene annotation, and biophysics are enabling a renewed focus on the synthesis of milk lipids within the mammary gland. As structures are identified, they are being assigned novel functions within the infant. Saturated and monounsaturated fatty acids are becoming recognized as metabolic signal molecules acting systemically. The complex composition of human milk lipids directs a spontaneous self-assembly process during digestion creating structures at the nanometer length scale that enhance absorption of a wide range of nutrients.

Assembling Lipid Particles in the Mammary Epithelia

The assembly of complex lipids into highly organized bilayer membrane structures is the central organizational and energetic paradigm of biology. However, lipids provide a variety of other structural assemblies to higher organisms in
particular as transport. The milk fat globule is one of biology’s more prodigious lipid transport systems. The particles themselves are highly disperse with sizes spanning three orders of magnitude and vary with stage of lactation, health of the mother, etc., with the average diameter varying between 1 and 10 μm [2]. The fat globule is a core of triacylglycerides assembled within the endoplasmic reticulum (ER) of the mammary epithelial cell from fatty acids of dietary, adipose depot, and lipogenic origins. This core of triacylglyceride is coated by a single monolayer of phospholipids derived from the ER membrane [3]. The milk fat globule is unique in containing also an intact bilayer membrane, which enrobes the globule as it exits the epithelial cell. This membrane as a result is composed of the lipids and proteins of the epithelial cell plasma membrane, including significant quantities of cholesterol, phosphatidylcholine, and sphingomyelin [4, 5]. Research has documented that these complex lipids have effects on composition and function of tissues literally from intestine to brain [6–8]. Yet one of the most important determinants of both composition and structure, globule size, is poorly understood. New research methods and models are making it possible to interrogate the more dynamic and complex lipid structures that are integral to the structures and functions of milk lipids.

The basic steps of globule assembly in the mammary epithelia are described [9]. Triglyceride synthesis by enzymes within the ER gives rise to an inert lipid phase of triglycerides bounded by a monolayer of ER phospholipids that is released into the cytoplasm as a spherical lipid particle. What happens after their formation is both the most unique property of milk fat globules and their least understood. Globules migrating towards the apical membrane fuse into larger discrete particles, and then the membrane and particle surfaces become enriched in key proteins notably xanthine oxidase and butyrophilin. At this stage, the particles’ outer surface interacts with the inner face of the plasma membrane, and the entire particle with surrounding plasma membrane is excreted as an intact milkfat globule.

The most important determinants of globule size are the flux of triglycerides into the nascent particle within the ER, their release into the cytosol and the subsequent fusion (or not) of these particles prior to attachment with the plasma membrane and secretion. Triglyceride flux is driven by the substrate fatty acid availability and the net energy charge within the cell. Breakthrough new research demonstrates that these same determinants are responsible for particle fusion [10]. Central to these breakthroughs is the realization that specific fatty acids are more than simple substrates to triglyceride formation, they influence the metabolic pathways and biophysical events that guide particle formation, composition, and fusion (Fig. 1). Lipid synthesis is metabolically controlled in part by the PGC1 family of transcription coactivators that drive the expression of the concert of genes necessary to assemble mitochondria and synthesize lipid particles [11]. PGC1 is
upregulated in the mammary epithelia by palmitic and oleic acids yet asymmetrically. Palmitic acid enhanced triglyceride and phosphatidyl choline concentration. Oleic acid enhanced triglyceride production and PGC1 activation and mitochondrial biosynthesis with a net increase in phosphatidyl ethanolamine concentration. This difference in phospholipid composition has an important effect on particle fusion events. As a membrane constituent, phosphatidyl ethanolamine is fusagenic. Thus, with these insightful breakthroughs in the basic mechanisms of particle fusion and the metabolic production of fusagenic phospholipids, guiding globule size distribution is becoming an accessible mammary gland outcome.

**Milk Fat Globule Disassembly**

To date, little research has addressed the implications of the structural dimensions of human milk fat globules within the intestine of the infant. The retention of milk’s lipid production through the evolution of lactation in mammals im-

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**Fig. 1.** Assembly of lipid globules within the mammary gland. Fatty acids with distinct metabolic activities and their consequences to biophysical events are indicated. C16:0, palmitic acid; C18:1, oleic acid; PGC1, PPARγ coactivator 1; PS, phosphatidyserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Tg, triglyceride. Modified from Cohen et al. [23].
plies that this remarkable structure is of considerable functional value to the mother-infant pair [1]. However, the techniques necessary to describe the structures of lipids in dynamic detail have not been available [4]. New physics technologies that are capable of interrogating the complex structures of lipids as the nanometer length scale and the time dimension of digestion are emerging [12].

While the spontaneous assembly of phospholipids into bimolecular membranes is the signature feature of lipid evolution, complex lipids spontaneously form a wide variety of three-dimensional structures from membranes to micelles to cubic phases [13]. These observations of purified lipids have been difficult to translate into biological mechanisms however. Why is the physical science not been taken to the biological dimension? The very nature of the forces underlying the spontaneous, rapid assembly of lipids into ephemeral, diaphanous structures is the reason the traditional experimental strategies of biochemistry are so disabled. The tools that worked so well on the “rugged” molecules of biology – proteins, polysaccharides, and polynucleotides – are insufficient in characterizing the soft structures of lipids. This is changing with the emergence of techniques designed to capture structural information on the length and time scales relevant to biological structures. One of the more actionable targets of lipid structures is the development of drug delivery systems tailored to the poor solubility of many therapeutically attractive but poorly bioavailable drug candidates [14]. The ultra-high intensity of coherent X-rays emerging from synchrotron sources enables techniques such as synchrotron small angle X-ray scattering to obtain diffraction patterns over much shorter time frames. This speed of diffraction measurement is capable of capturing transient lipid phases during digestion [15]. The pharmaceutical question is whether complex lipid phases can enhance drug delivery. It is also possible to ask the basic mechanistic question: do different lipid mixtures form distinct phases as they undergo the hydrolytic reactions of lipid digestion? By going biological instead of chemical, Ben Boyd’s group answered both questions at once [16, 17]. While the majority of lipid mixtures exhibit the appearance of micellar and lamellar phases during real time digestions, only milk lipids have produced a transient but highly robust signal associated with an extensive cubic phase. The implications of the formation of cubic phases during digestion are profound to the processes of nutrient absorption. Cubic phases are bicontinuous three-dimensional structures. The nature of the cubic phase means that there is effectively both a water phase and a lipid phase existing simultaneously as the continuous phase providing an unrestricted path for free diffusion across extended-length scales for both water-soluble and lipid-soluble nutrients within the gut. The remarkable properties of these complex phases are their thermodynamic spontaneity. The formation of cubic phases with definable three-dimensional structure at the nanometer-length scale, extending for millimeters, does not re-
quire apparatus, energy, or template. These phases are the consequence of the composition of the lipids themselves, and they form spontaneously (Fig. 2). Strikingly, the complex lipids of milk form cubic phases during digestion, whereas the lipid mixtures found in formula do not [16].

Lipids as Metabolic Signals

The dossier of lipid molecules documented to exhibit a signaling function and trigger and/or regulate cellular processes continues to grow. Many of these follow classic pathways in which protein receptors bind to different lipids transducing cellular events downstream into cellular processes [18, 19]. The oxylipins are lipid signals of local stress. The essential n-6-polyunsaturated fatty acids and in particular arachidonic acid have been demonstrated to provide these signaling functions [20].

Lipids signal to a wide range of biological processes. Their interaction with fuel metabolism and transport may be of particular importance to the development of metabolic regulation within infants. Although the majority of scientific research has focused on the essential fatty acids that must be obtained within the maternal diet, actions of the fatty acids made within the mammary gland are emerging as important as signaling molecules.

Palmitic acid is the end product of fatty acid synthesis and is ubiquitous throughout biology. Palmitic acid is: a constituent fatty acid in cellular membranes; a fuel, producing 106 moles of ATP energy via beta oxidation; is acylated to various membrane proteins; and is a major component of storage lipids.
Now to this list must be added signaling. Puigserver and Spiegelman [21] discovered the functions controlled by a protein complex that they termed the PPAR gene transcription coactivator (PGC-1α). This transcription factor coactivator assembles protein regulatory units in the nucleus to control mitochondrial biogenesis. This laboratory made an equally important realization that when exposed to high levels of palmitic acid, liver cells both in vivo and in vitro actively turned on a closely related protein, PGC-1β [19]. PGC-1β simultaneously controlled fatty acid synthesis, triglyceride assembly, phospholipid synthesis and cholesterol biosynthesis. Their work identified the molecular switch linking dietary saturated fat and serum cholesterol as PGC-1β, and its most potent ligand is palmitic acid. Thus, palmitic acid is the signal activating PGC-1β and its cellular functions. By this transcription mechanism, the liver regulates the net production of very-low-density lipoproteins (VLDL) and their molecular constituents. Palmitic acid acts as a hepatic signal to stimulate VLDL synthesis and assembly. This specific signaling action in the liver and to VLDL production may be important both in infancy and throughout life.

Palmitoleic acid (16:1ω7) is formed by the desaturation of palmitic acid by the stearoyl CoA desaturase enzyme. In practice, palmitoleic acid is produced when the enzymes of fatty acid synthesis and stearoyl CoA desaturase are more active than the elongase enzyme converting palmitic to stearic acid. This is typically true during active lipogenesis in humans and other animals. Cao et al. [22] identified and made a bold prediction characterizing palmitoleic acid as a novel lipokine, or lipid hormone involved in regulating the insulin-sensitizing effects of diet. Their studies are a demonstration of palmitoleate as a lipid signal, and a unifying model of metabolic products acting to control whole body fuel metabolism.

These are examples of signaling systems based on non-essential fatty acids regulating cellular, tissue, and whole body functions. Each of these lipid signals is conspicuously present in human milk. In view of these potentially immediate benefits of dietary lipids in infant health, it seems prudent to consider the complexity of lipids within human breast milk as an important asset that pursuing more simplified compositions puts at risk.

**Conclusions**

Lipids in milk are highly diverse, and their retention throughout evolution is a compelling story of biological structure and function. Though the research is not complete, principles can be taken forward in considering optimal dietary lipid compositions and structures for infancy. The biosynthesis of fatty acids and complex lipids within the mammary gland is a relatively variable series of pro-
cesses depending on genetics, diet, maternal physiology, and metabolic status. This variability poses both a challenge and an opportunity. Understanding how globules are synthesized and assembled has proven to be a daunting task. Nonetheless, as the basic mechanistic principles are emerging, opportunities are also emerging to guide these processes towards more healthy, protective, and functional lipid systems. Similarly, the diversity of composition is revealing a diversity of functions within the mammary gland and the infant. The roles of lipid molecules in signaling are proving to be astonishingly diverse. Whereas lipid signaling was once thought to be a simple response to stress via the oxygenation of polyunsaturated fatty acids, a host of lipid molecules are now implicated in normal metabolism, physiology, immunology, and even cognition and behavior.

Finally, lipids are a unique class of biomolecules because of the structures that they make within and outside cells. The evolution of mammalian milk has retained the unique fat globule structure across marsupials and all mammals. Finally, new biophysical tools are beginning to assign specific properties to the structural dimension of dietary lipids. Once again, biology is teaching chemistry and pharmacology how best to guide complex processes to desired outcomes.

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References

Carolyn Slupsky provided an overview of the principles and applications of the omics toolset from genomics to metabolomics and their investigative power in biological research. She highlighted in turn the need for such approaches in milk research from the composition of milk and the health of infants to the establishment of normal gut colonization of the newborn in the prevention of the development of disease. She then provided vivid examples of that power illustrating that milk is more than just a nourishing biofluid, it is a metabolic system containing highly enriched metabolites whose presence and functions have not been recognized by traditional experimental designs. It was her research comparing metabolic profiles of infant primates fed either human milk or formula that was revolutionizing our understanding of milk’s importance. Carolyn Slupsky showed that infants are unique relative to adults in the presence and abundance of various metabolites. She further mapped these differences to metabolic pathways, and this powerful integrative approach made it abundantly clear that milk propels the entire metabolic development of infants.

Kevin Nicholas placed lactation within the context of mammalian evolution providing a temporal map of lactation’s progress from pre-mammals to humans. He identified various comparative approaches that illustrate the power of comparative experimental strategies across Mammalia in the discovery of structure and function relationships in milk. Then, he revealed the fascinating variations
in lactation strategies matched to the variations in gestational age of different mammals. As milk researchers, we forget just how diverse mammalian gestation can be and how milk has adapted to the wide range of birth maturities with compositional features matched to the different developmental trajectories of different mammalian infants. But he saved the best for last. Kevin Nicholas then revealed a brilliant vision for an entire discovery platform for intervention in human health. He described this platform in which unique biological activities are discovered in marsupials, mapped to bioactive components of milk and their cognate targets in infants, identifying their mechanisms of action and dose dependency. He then revealed a biotechnology pipeline in which these discoveries are rapidly translated into stepwise production systems bringing therapeutic interventions to practice for premature infants, clinical patients, even the elderly.

In the final talk of the session, J. Bruce German reviewed the current state of knowledge of the milk fat globule, its complexity and its diversity across Mammalia and among human mothers. He then began a voyage from the globule formation to its digestion highlighting the structural assembly processes within the mammary gland and the infant intestine. The tools of biophysics are revealing how composition of milk lipids guide the spontaneous self-assembly of globule components within the mammary gland and the self-assembly of unique three-dimensional phases within the intestine of infants. Because these structural transformations are driven by spontaneous thermodynamics rather than explicit apparatus, it is the composition of lipids that is central to their success. Breakthrough research by Nurit Argov identified specific phospholipids responsible for the fusion of lipid particles in the mammary epithelial cell and their importance to final lipid globule size within milk. Breakthrough research by Ben Boyd similarly identified the formation of cubic phases during the digestion of milk is unique to milk lipids and not achieved by simple emulsions. Milk composition thus guides the formation of lipid structures within the infant that ensure the absorption of the diverse lipid components throughout milk. Finally, J. Bruce German highlighted the metabolic importance of the fatty acids whose abundance and absorption are the result of this milk complexity. Fatty acids synthesized within the mammary gland and not previously considered important in fact, guide whole-body lipid metabolism within the infant.

This session provided a view of the remarkable breadth of science now being recruited to the goal of understanding human milk, its compositions, structures, and functions. We emerge with a renewed commitment to the importance of milk to the early nourishment of infants and the power of research to discover the intimacy of human development and its relationships to diet.

J. Bruce German
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