Human Milk: Lessons from Recent Research

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*Natalia Wagemans, MD*
Head of
Nestlé Nutrition Institute
Vevey (Switzerland)
Human milk is the best source of nutrients both for low-birth-weight (LBW) infants and healthy term infants. For LBW infants, the availability of human milk often is a problem, in particular during longer periods of hospitalization immediately after birth. Because LBW infants fed human milk have lower infection rates, the supply in hospitals must be improved. Breastfed term infants in particular those in developing countries experience fewer and shorter infection periods. When compared to formula-fed infants, term infants fed human milk have different gut microflora, exhibit different growth patterns, and even face a lower long-term risk of chronic diseases, such as obesity, types 1 and 2 diabetes, and cardiovascular disease. During recent years, it has become clear that human milk provides both optimal nutrient supply and functional ingredients, such as proteins, lipids, and oligosaccharides, which can contribute to short- and long-term health outcomes. Although the composition of infant formulas has evolved with increasing knowledge of human milk, differences in outcomes between breastfed and formula-fed infants are still observed. Efforts to improve the composition of infant formulas are complicated because human milk and its key components change continuously over time. Consequently, narrowing the gap between human milk and infant formula requires a deep understanding of how the quantity and quality of key nutrients change over time.

Haiden and Ziegler [this issue, pp. 8–16] review breast milk banks in hospitals which play an essential role by providing human milk to those infants who would otherwise not be able to receive it. Human milk helps to protect LBW infants from necrotizing enterocolitis and from sepsis. Milk banks collect, screen, pool, store, process, and distribute human milk according to standardized procedures. Donating women are carefully selected and tested for communicable diseases. Although heat treatment of human milk diminishes anti-infective and other beneficial properties, for LBW infants, donor milk is still highly preferable in comparison to formula. The network of human milk banks at present is already well developed in South America, Europe, and South Africa.

Protein ingested with breast milk provides all indispensable amino acids which are necessary for new protein synthesis for growth and replacement of losses. Haschke et al. [this issue, pp. 17–27] mention that protein concentrations in breast milk are highest during the first months when daily weight gain and protein needs for growth are also highest. LBW infants have higher protein needs than term infants and need protein supplements when fed human milk. Based on our better understanding of protein evolution in human milk during the stages of lactation, new infant formulas with lower concentrations of high-quality protein according to the infant’s needs have been created, successfully tested, and are now available in
many countries. The authors also review the bioactive proteins in human milk and their functions which can be broadly classified into 4 major categories, that is, providing protection from microbial insults and immune protection, aiding in digestive functions, supporting gut development, and being carriers for other nutrients. Indeed, some proteins like lactoferrin and sIgA have been extensively studied for their biological functions also in infants, whereas others may require more data in support to further validate their proposed functions.

Koletzko [this issue, pp. 28–41] focuses on functional lipid components in human milk. Adding complex lipids and milk fat globule membranes as found in human milk to vegetable oil-based infant formula has the potential to enhance infant development and reduce infections. Cholesterol provision with breastfeeding modulates sterol metabolism early in life and may induce long-term benefits such as lowering cholesterol levels later in life. The long-chain polyunsaturated fatty acids docosahexaenoic (DHA) and arachidonic (ARA) acids have been extensively studied during the last 3 decades. Recent gene-diet interaction studies indicate that breastfeeding, which provides DHA and ARA, improves cognitive development and reduces asthma risk at school age, particularly in those children with a genetically determined lower activity of DHA and ARA synthesis. It appears prudent to follow the biological model of human milk lipids as far as feasible when future infant formulas are designed.

In addition to bioactive proteins and lipids, breast milk contains a variety of carbohydrates—human milk oligosaccharides (HMOs)—that protect the newborn and stimulate innate and adaptive immune development. Donovan and Comstock [this issue, pp. 42–51] review the role HMOs play in neonatal gastrointestinal and systemic immune development and function. Studies have shown that human milk contains a higher concentration, a greater structural diversity, and a higher degree of fucosylation than the milk oligosaccharides in other species, particularly bovine milk. The commercial availability of large quantities of certain HMOs has allowed studying the functions of specific HMOs, which include protecting the infant from pathogenic infections, facilitating the establishment of the gut microbiota, promoting intestinal development, and stimulating immune maturation. Two HMOs, 2′-fucosyllactose (2′FL) and lacto-N-neotetraose (LNnT), have recently been added to infant formula. The authors point out that this is an initial step to narrow the compositional gap between human milk and infant formula, but it is still not clear whether 1 or 2 HMOs will recapitulate the complexity of actions exerted by the complex mixture of HMOs in breast milk. Adding HMOs to formulas, however, is a big step forward compared to the addition of the first-generation ‘prebiotics’ to formula more than a decade ago.

The new insights on functional components in human milk confirm their influence on gut development and microbiota, immune development and response, and growth. They further support present international recommendations that all infants should be breastfed beyond 6 months and LBW infants should have access to human milk from the first day of their enteral nutrition. Infant formula producers are challenged to further narrow the gap to human milk.

Ferdinand Haschke
Human milk exerts strong trophic effects on the infant gut and thereby enables full enteral feedings to be reached earlier than without human milk

**Key insights**
Human milk has a protective effect for premature infants who are at risk of necrotizing enterocolitis and sepsis, two medical conditions associated with high rates of mortality. Milk banks collect, screen, store, process, and distribute human milk when needed. The strict quality control procedures followed by milk banks ensure the safety of donor milk while retaining many of the beneficial effects of raw human milk.

**Current knowledge**
There are many circumstances where milk from the infant’s own mother is not available or sufficient. Milk banks function as repositories of donated milk. The most common practice is to pool milk from multiple donors, in order to ensure an even distribution of nutrients such as protein and fat. Donated milk is often pasteurized using the Holder process and frozen for up to 1 year. This method of pasteurization offers a good compromise between microbiological safety and biological quality. The typical milk donor is of average childbearing age, with a milk supply large enough to allow milk donation while meeting her own infant’s needs. Donors are screened for general health, including for recreational drug use and infectious diseases such as viruses and syphilis.

**Practical implications**
Premature infants represent the largest group of recipients of donor milk. Due to their high risk of infection and necrotizing enterocolitis, premature infants also derive the greatest benefits from receiving human milk. Worldwide, there is growing interest in human milk banking, with efforts to introduce milk banks even in third world regions. Guidelines from international pediatric societies indicate that if the mother’s own milk is not available, donor milk should be the next choice. Human milk should be stored in established banks that adhere to strict safety guidelines.

**Recommended reading**
Human Milk Banking

Nadja Haiden\textsuperscript{a}  Ekhard E. Ziegler\textsuperscript{b}

\textsuperscript{a}Division of Neonatology, Pediatric Intensive Care Medicine and Neuropediatrics, Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Vienna, Austria; \textsuperscript{b}Department of Pediatrics, University of Iowa, Iowa City, IA, USA


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Introduction

It is probably not widely appreciated that human milk banking is an absolute necessity if all infants are to enjoy the benefits of human milk. This is so because a substantial number of infants, especially premature infants, are unable to receive adequate amounts of their mothers' milk for a variety of reasons. Were it not for milk banks, these infants could not be fed human milk and would suffer the consequences. Premature infants derive very important protections from human milk.

Unfortunately, there are circumstances where milk from the infant’s own mother is not available. Milk donated by other women (donor milk) must then fill the gap. Premature infants constitute the largest and most important group of infants where milk from other women is needed because their own mothers’ milk is not avail-

Keywords

Human milk banking · Donor milk · Milk processing · Pasteurization · Preterm infants · Sick infants

Key Messages

- Donor milk from human milk banks is essential for premature and sick infants.
- Milk banks strictly follow guidelines for the storage, processing, and handling of human milk to ensure the safety of donor milk.
- Pasteurized donor milk retains many of the beneficial health effects of raw human milk.

Human milk banks play an essential role by providing human milk to infants who would otherwise not be able to receive human milk. The largest group of recipients are premature infants who derive very substantial benefits from it. Human milk protects premature infants from necrotizing enterocolitis and from sepsis, two devastating medical conditions. Milk banks collect, screen, store, process, and distribute human milk. Donating women usually nurse their own infants and have a milk supply that exceeds their own infants’ needs. Donor women are carefully selected and are screened for HIV-1, HIV-2, human T-cell leukemia virus 1 and 2, hepatitis B, hepatitis C, and syphilis. In the milk bank, handling, storing, processing, pooling, and bacterial screening follow standardized algorithms. Heat treatment of human milk diminishes anti-infective properties, cellular components, growth factors, and nutrients. However, the beneficial effects of donor milk remain significant and donor milk is still highly preferable in comparison to formula.

Preterm infants · Sick infants

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able or is not available in sufficient quantity. Human milk banks collect, screen, pasteurize, and distribute donated breast milk to hospitals or outpatient recipients [1]. Usually the collection, storage, and processing in a human milk bank follows established guidelines. Milk banks are by far the most important providers of donor milk, the fact not withstanding that other venues of milk donation are also used.

**History**

The first human milk bank was founded in 1909 in Vienna, Austria. Wet nursing was widely practiced in Europe during the 19th century in order to provide human milk for infants whose mothers were unable to provide milk for their infants. However, wet nurses were not always available or, when available, pursued unhealthy lifestyles or carried infections that could be transmitted through milk. An alternative to wet nursing was found in human milk banking.

Shortly after Vienna, the first milk bank opened in the United States in the Boston Floating Hospital and many others followed all over the world. In the 1960s, efforts in human milk banking faded due to advances in neonatal medical care and infant nutrition, mainly the development of high-quality infant formulas. In the 1980s, the new infectious disease, HIV, arrived. As it is transmissible through breast milk, this led to the closing of many milk banks. Once disease transmission via human milk was recognized as a health hazard, serological testing of the mother became necessary. The added financial burden drove some milk banks out of business. Appropriate screening of donating mothers as well as adherence to standards of procedure have reversed that trend since the early 2000s.

Milk banking activity varies greatly between different parts of the world due to a variety of reasons: sometimes the reasons have to do with economics and funding, and sometimes they are linked to religious and cultural factors. Globally, there is an increase of interest in milk banking all over the world. Currently there is a move to open many milk banks in India and other Asian countries such as Vietnam, China, and Japan. The increase of interest goes along with the recommendations of large pediatric societies, such as ABM, ESPHGAN, and AAP, to promote human milk feeding in premature infants [2–4]. All guidelines say that the mother’s own milk is the first choice for an infant. However, if the mother’s own milk is not available, donor milk is the recommended alternative. A further important recommendation is that donor human milk should be provided by an established human milk bank that follows standard safety guidelines.

Table 1 gives an overview about the established and planned milk banks all over the world.

**Why Is Human Milk Being Banked?**

The main function of milk banks is to serve as repositories of donated milk so it is available when needed. Milk banks receive milk from donors, process it, and store it until used. Most commonly milk from multiple donors is pooled, although some banks pool milk only of individual donors (single-donor banks). Usually, milk provided by milk banks has undergone pasteurization. Once pas-
teurized, milk is placed in small (100–150 mL) containers and is stored frozen for up to 1 year depending on local guidelines. In the US, milk banks charge a processing fee to cover the costs of collecting and handling of the milk. The type of container affects the stability of the constituents of human milk, while colostrum was reported to remain stable when refrigerated for 24 h in any container (Table 2) [5].

### Who Are the Milk Donors?

Most milk is donated by women, who have nursed their own infant for some time and realize that their milk supply is large enough to allow them to donate milk while still satisfying their own infant’s needs. A study from France [6] showed that the typical donating mother was of average childbearing age with strong support at home. Almost half did not work outside of the home, and a large number were from the health and social services fields. Reasons for donation were largely altruistic, and a general optimistic attitude prevailed within the mothers.

To be eligible as milk donors, women must not be using recreational or other drugs, and their physician as well as the infant’s physician must agree that milk donation is in order. The milk bank will obtain a health history and obtain blood for testing. Usually, the donating mother is screened for HIV-1, HIV-2, human T-cell leukemia virus 1 and 2, hepatitis B, hepatitis C, and syphilis [7]. If all requirements are met, the donor is provided a supply of milk containers and receives instruction as to the appropriate means of milk expression. The donor obtains milk by mechanical pump or manual expression and stores it in the freezer compartment of their home refrigerator before delivery to the milk bank. The milk is transported to the milk bank either by the mother herself or by a transport service provided by the milk bank. It is important that the cooling chain is never interrupted; therefore, special cooling bags or cooling boxes have to be used during transportation from home to the milk bank.

Milk donation is an act of unselfishness. In most countries donors receive no compensation, but in some countries donors receive modest monetary compensation for actual costs incurred. It represents mostly a token of appreciation.

### How Is Milk Handled by the Bank?

Milk banks generally follow standardized procedures for the collection and handling of donated milk [7]. Donors are instructed by the milk bank about recommended breast cleaning and breast pumping procedures. The bank provides containers for milk. Pooling of milk from several pumpings is often performed. Each container must carry the name, date, and time of expression. The milk remains in the freezer until it is delivered to the bank. Figure 1 shows the human milk banking process.

At the bank, milk is stored at –20 °C. On the day before processing, the donated milk is placed in a refrigerator for overnight thawing. On the day of pasteurization, the milk from 3–5 donors is pooled. Pooling serves the purpose of distributing nutrients, such as protein and fat, as well as foreign substances evenly. After pooling, the milk is placed in individual 100 mL bottles. Pasteurization is carried out in a water bath at 62.5 °C for 30 min followed by rapid cooling. Milk bottles are then stored at –20 °C until use of the milk. This method (Holder pasteurization) is widely felt to represent a good compromise between microbiological safety and nutritional/biological quality of donor milk [8]. Nevertheless, methods that lead to less nutrient loss and, perhaps, are less time consuming would be desirable and are being sought [9].

1. High-temperature short-time pasteurization at 72 °C for 5–15 s is such a method. It reaches a better compromise between microbiological safety and nutri-

### Table 2. Effect of container type on milk constituents (adapted from Lawrence and Lawrence [18])

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Pyrex</th>
<th>Polypropylene</th>
<th>Polyethylene bags</th>
<th>Polyethylene (rigid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>stick to glass</td>
<td>maintain phagocytosis</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fat-soluble vitamins</td>
<td>no effect</td>
<td>no effect</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>no effect</td>
<td>no effect</td>
<td>lower</td>
<td>stable</td>
</tr>
<tr>
<td>Secretory IgA</td>
<td>–</td>
<td>–</td>
<td>very easy spill</td>
<td>yes</td>
</tr>
<tr>
<td>Difficult to handle</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>no</td>
</tr>
<tr>
<td>Recommended for milk</td>
<td>highly</td>
<td>no</td>
<td>–</td>
<td>yes</td>
</tr>
</tbody>
</table>

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Haiden/Ziegler
The method is not yet used routinely due to lack of suitable instrumentation.

2. The combination of ultrasound and heat (thermoultrasoundic treatment) is an emerging technique that allows milk to retain more of its bioactive components compared with thermal pasteurization [14]. However, the current experimental system is limited to small volumes and needs to be scaled up.

3. High-pressure processing (HPP) shows promise as an alternative to pasteurization. Total immunoglobulin A immunoreactivity and lysozyme activity are significantly higher after HPP compared with pasteurization [15]. Besides, HPP is faster and probably more convenient than Holder pasteurization. It seems a promising technology, but further investigation is necessary before it can be used routinely.

4. Finally, there is Ohmic heating, a new technology under investigation. Ohmic treatment is a thermal processing method wherein the food material, which serves as an electric resistor, is heated by passing an electric current through it, which leads to rapid and uniform heating. Like thermal processing, Ohmic heating inactivates microorganisms by heat. The first experimental trials have shown no modification of the protein pattern of milk at a temperature of 72°C and only small changes at a temperature of 78°C.

In some countries, the milk is tested bacteriologically before it is pasteurized, in some countries after pasteurization, and in others the milk is tested before and after pasteurization. Some countries, such as Norway, have a tradition of feeding raw donor milk. In Norwegian milk banks, each container of milk from a donor is screened for bacteria. Milk that contains any pathogens or high counts (>100,000 colony-forming units/mL) of any other bacteria is destroyed. Milk with a low bacterial count (<10,000 colony-forming units/mL) is used for the smallest preterm babies [16]. Ronnestad et al. [17] described an incidence of late-onset sepsis in a Norwegian cohort of extremely low-birth-weight infants receiving raw breast milk or donor milk of 19.8% (80/405), which was similar to the incidence in the Vermont Oxford quality network (21.4%, in the year 2000). Therefore, it is unlikely that microbiologically screened raw milk is hazardous for preterm infants.

Who Are the Recipients of Donor Milk?

The most common recipients of donor milk are the following [18]:

- Premature infants, especially infants with a birth weight below 1,500 g, because of their high risk of infection and necrotizing enterocolitis
- Infants with gastrointestinal anomalies undergoing gastrointestinal surgery leading to short bowel syndrome
- When the mother is temporarily unable to nourish her infant completely, e.g. when the mother is ill or hospitalized
- Weaning from parenteral nutrition
- Metabolic disorders, especially amino acid disorders
- Before the mother’s own milk comes in (first few days after birth).

Premature infants are not only the largest group of recipients of donor milk, they are also those who derive by far the greatest benefits from receiving human milk. Human milk exerts strong trophic effects on the infant gut and thereby enables full enteral feedings to be reached earlier than without human milk [19]. Human milk protects premature infants strongly against necrotizing enterocolitis [19, 20] and against sepsis [21], two conditions that carry high mortalities. Some mothers object intuitively to the use of donor milk, which is why donor milk is fed only after its source has been explained to the mother and she has agreed to its use.
The reason why mothers of premature infants are often not able to provide milk at all or provide milk only in insufficient quantity is that premature delivery, by shortening pregnancy, foreshortens the period of preparatory lactogenesis. Also, the necessary mechanical milk expression is less effective in stimulating and maintaining milk production than suckling by a mature infant.

It has been suggested that the availability of donor milk could act as a disincentive to mothers to provide milk for their premature infants. There are indeed data in the literature that support this contention [22]. However, a 1-year study in 2010 involving all NICUs in Italy showed that the rate of exclusive breastfeeding at the time of discharge was significantly higher in NICUs with milk banks than in NICUs without milk banks (29.6 vs. 16.0%) [23]. This confirms anecdotal reports from other areas. It appears thus that the availability of donor milk has a positive effect on the motivation of mothers to provide milk for their infants.

Donor milk is also fed to older babies and to children with a variety of medical conditions, including severe food allergy or feeding intolerance, growth failure while on formula, intractable rotavirus enteritis, and during chemotherapy for cancer [24]. Occasionally, adopted infants receive donor milk. Furthermore, there are several case reports where donor milk was used in adults with special medical conditions, e.g. in liver transplanted patients who were IgA deficient to supply extra IgA [25] or in adult cancer patients [26].

**The Composition of Donor Milk**

It is widely appreciated that the composition, in particular the protein and fat content, of individual expressions of human milk varies greatly. This has led to the promotion of bedside human milk analyzers and procedures for nutrient fortification of individual milk samples. However, with donor milk the variability of composition is greatly reduced due to pooling. Milk from multiple pumpings is usually pooled by the donor mother before delivery to the bank. Pooling of milk from multiple donors is then performed by the milk bank, with the result that the protein and fat content of pooled milk is quite stable and predictable. Michaelsen et al. [27] reported that fat and protein concentration vary widely from sample to sample but that variability decreases sharply with pooling of samples from multiple donors. At the Mother’s Milk Bank of Iowa, the nutrient content of 37 milk pools collected over a period of 2 years (2003-2005) was analyzed. The (true) protein concentration averaged 8.22 g/L with an SD of 0.59 g/L, and the fat content averaged 39.0 g/L with an SD of 3.51 g/L. The variability of composition is thus far lower than that between individual samples [28]. Low variability is of particular advantage in the case of premature infants because variability of the protein content is often the source of concerns about inordinate-high intakes of protein. Low variability of composition of donor milk has the advantage that the infant’s nutrient intake varies little from feeding to feeding and is essentially always known, whereas with the mother’s own milk the nutrient content may vary greatly from feeding to feeding.

### Table 3. Effect of pasteurization on milk components (adapted from Arnold [1])

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immune components</strong></td>
<td></td>
</tr>
<tr>
<td>C3 complement</td>
<td>0%</td>
</tr>
<tr>
<td>IgA</td>
<td>0–150%</td>
</tr>
<tr>
<td>IgG</td>
<td>0–82.8%</td>
</tr>
<tr>
<td>IgM</td>
<td>0%</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0–123%</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0–393%</td>
</tr>
<tr>
<td><strong>Cellular components</strong></td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>number decreased, 0% functionality</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>number decreased, 0% functionality</td>
</tr>
<tr>
<td><strong>Enzymes, growth factors</strong></td>
<td></td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td>61.8% completely destroyed</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>completely destroyed</td>
</tr>
<tr>
<td>Bile salt stimulated lipase</td>
<td>completely destroyed</td>
</tr>
<tr>
<td>Esterase</td>
<td>93.9%</td>
</tr>
<tr>
<td>Transforming growth factor α</td>
<td>99% whey decreased relative to fat</td>
</tr>
<tr>
<td>Transforming growth factor β&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Whey:casein ratio</td>
<td></td>
</tr>
<tr>
<td><strong>Nutrients</strong></td>
<td></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>94–100%</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>103%</td>
</tr>
<tr>
<td>Folic acid</td>
<td>65–95%</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>65–85%</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>77–94%</td>
</tr>
<tr>
<td>Biotin</td>
<td>102–110%</td>
</tr>
<tr>
<td>Niacin</td>
<td>100–106%</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>93–98%</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;</td>
<td>85–93%</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>64–94%</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>103%</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>106%</td>
</tr>
<tr>
<td>Zinc</td>
<td>redistribution of zinc pattern</td>
</tr>
</tbody>
</table>

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Is Donor Milk as Good as Mother’s Milk?

The fact that, with some exceptions, donor milk undergoes pasteurization has led to concerns that some or all of the protective effects of human milk may be lost. Studies assessing milk components before and after pasteurization have documented that indeed several important components of human milk are reduced in concentration or are eliminated altogether, as summarized in Table 3. Heat treatment affects anti-infective and cellular components, growth factors, and some nutrients, depending on the heat and duration of exposure. Enzymes are most heat-sensitive while immune components are compromised but not completely destroyed.

Processing of human milk also affects unsaturated fatty acids [29] and damages the membrane of milk fat globules [30]. Human milk contains stem cells with multilineage properties and variable expression of pluripotency genes normally found in human embryonic stem cells [31]. It is likely that these stem cells are destroyed during heat treatment. On the other hand, some important protective components such as the oligosaccharides are essentially resistant to the effects of heat.

Given these effects of high-temperature processing, it would be expected that the protective effects of human milk might be diminished but not abolished altogether. That is exactly what the literature shows. In 5 trials comparing formula with donor milk with regard to the incidence of necrotizing enterocolitis, the risk of necrotizing enterocolitis was nonsignificantly diminished in each trial. However, collectively, the 5 trials showed a significant protective effect of donor milk compared to formula (Fig. 2) [32]

A direct comparison of fresh against pasteurized human milk performed by Narayanan et al. [33] showed a somewhat reduced protective effect against infection (14.3 vs. 10.5% infection) which was still much stronger than the effect of formula (33.3% infection). It is thus evident that the beneficial effects of pasteurized human milk are diminished vis-à-vis fresh milk but that enough of the protective effects remain to render donor milk the feeding of choice for premature infants in the absence of any or sufficient maternal breastmilk.

Is Donor Milk Safe?

Because of the potential for transmission of disease pathogens, sometimes there are concerns about this possibility. With current donor screening and pasteurization of donor milk, the possibility of disease transmission is infinitesimally small. In fact, there is not a single case of documented disease transmission through banked donor milk in recent decades. Whether that can also be said about informal milk exchanges is not known.

Is Donor Milk Cost-Effective?

Because milk banks charge a processing fee ($6–7/100 mL donor milk), it has been asked whether the benefits accruing to the infants justify the expense. Although it is inappropriate to ask this question in relation to deadly conditions (necrotizing enterocolitis, sepsis), fortunately several studies have documented that the use of donor milk is cost-effective [34, 35]. Thus, the use of donor milk not only saves lives, it also saves the hospital money. It was reported that sometimes mothers buy breast milk for their premature or sick infants via the internet, social networks [36], from friends, from private providers, or in other informal sharing arrangements. In those cases, donor screening, quality control of the human milk, and shipping standards are missing. This behavior is risky and is not recommended.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Formula n/N</th>
<th>DBM n/N</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross (1983)</td>
<td>3/26</td>
<td>1/41</td>
<td></td>
</tr>
<tr>
<td>Lucas (1984a)</td>
<td>4/76</td>
<td>1/83</td>
<td></td>
</tr>
<tr>
<td>Lucas (1984b)</td>
<td>5/173</td>
<td>2/170</td>
<td></td>
</tr>
<tr>
<td>Liu (1999)</td>
<td>10/88</td>
<td>5/78</td>
<td></td>
</tr>
<tr>
<td>Schanler (2005)</td>
<td>1/44</td>
<td>0/37</td>
<td></td>
</tr>
<tr>
<td>Tyson (1983)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pooled RR:</td>
<td></td>
<td></td>
<td>2.46 (95% CI 1.19 – 5.08)</td>
</tr>
<tr>
<td>Heterogeneity:</td>
<td></td>
<td></td>
<td>$\chi^2 = 0.99 (p = 0.91); I^2 = 0.0%$</td>
</tr>
</tbody>
</table>

Fig. 2. Meta-analysis of trials comparing feeding of formula versus donor milk: effect on risk of necrotizing enterocolitis. DBM, donor breast milk. Adapted from Chauhan et al. [32] with permission.

All donor milk must be fortified with nutrients before it is fed to premature infants. In this regard, donor milk does not differ from the mother’s own milk. Most human milk fortifiers contain as protein source various fractions or derivatives of cow milk. One fortifier provides protein from human milk and is claimed to protect better against necrotizing enterocolitis than fortifiers containing bovine milk proteins, although evidence for that effect is lacking [37]. The lack of benefit, therefore, argues against use of the high-cost human milk-based fortifier.

Conclusion

Milk banks serve a vital function by providing human milk for premature infants who, for a variety of reasons, would otherwise not have access to human milk. As human milk confers major protective effects to premature infants, the availability of human milk is an important quality of care issue. The use of donor milk is widely endorsed [4, 38, 39].

References


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We thank Gillian Weaver, head of the European milk banking association EMBA, for her valuable input.

Disclosure Statement

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Recent findings on nutritive and bioactive proteins in breastmilk support the WHO recommendations that breastfeeding should be continued during the first year and beyond

Nutritive and Bioactive Proteins in Breast Milk
by Ferdinand Haschke et al.

Key insights
The protein intake of breastfed term infants has been used as the basis for estimating an infant’s protein requirements during the first year. Daily protein gain is highest in the very young infant and decreases rapidly in later infancy and in the second year of life. The protein content of breast milk evolves depending on the stage of lactation and time since delivery. Indeed, protein concentration in breast milk is high during the first few weeks of lactation and gradually subsides throughout the first year. The quantity and quality of breast milk is critical to support infant growth and long-term development.

Current knowledge
Proteins are the third most abundant solids found in breast milk. The variety of functions performed by the bioactive proteins and peptides in breast milk shed light on why breastfed infants have lower morbidity and fewer infections. Lactoferrin, secretory IgA, osteopontin, and various cytokines modulate the infant’s immune system alongside lysozyme, κ-casein, and lactoperoxidase, which have antibacterial functions. Other proteins regulate gut development and aid in the absorption of key nutrients.

Practical implications
Based on our better understanding of protein evolution in breastmilk across the stages of lactation, new infant formulas with lower protein concentration but better protein quality have been developed, tested, and made available in many countries. Low-birth-weight infants have higher protein requirements than term infants because of their higher daily protein gain per unit body weight. The concentrations of protein and amino acids in the breast milk of mothers who deliver preterm are higher during the first weeks of lactation compared to those of mothers who deliver at term. Supplementation of breast milk is needed to meet the high protein requirements of infants with very low and extremely low birth weight.

Recommended reading
Nutritive and Bioactive Proteins in Breastmilk

Ferdinand Haschke\textsuperscript{a} Nadja Haiden\textsuperscript{b} Sagar K. Thakkar\textsuperscript{c}

\textsuperscript{a}Department of Pediatrics, Paracelsus Medical University, Salzburg, and \textsuperscript{b}Department of Pediatrics, Medical University Vienna, Vienna, Austria; \textsuperscript{c}Nestlé Research Center, Lausanne, Switzerland

Abstract
Protein ingested with breast milk provides indispensable amino acids which are necessary for new protein synthesis for growth and replacement of losses via urine, feces, and the skin. Protein gain in the body of an infant is highest during the first months when protein concentrations in breast milk are higher than during later stages of lactation. Low-birth-weight infants have higher protein needs than term infants and need protein supplements during feeding with breastmilk. Based on our better understanding of protein evolution in breastmilk during the stages of lactation, new infant formulas with lower protein concentration but better protein quality have been created, successfully tested, and are now available in many countries. Besides providing indispensable amino acids, bioactive protein in breast milk can be broadly classified into 4 major functions, that is, providing protection from microbial insults and immune protection, aiding in digestive functions, gut development, and being carriers for other nutrients. Individual proteins and their proposed bioactivities are summarized in this paper in brief. Indeed, some proteins like lactoferrin and sIgA have been extensively studied for their biological functions, whereas others may require more data in support to further validate their proposed functions.

Key Messages
- Protein intake of breastfed term infants has been used as a model to estimate protein requirements during the first year. They are higher during the first months when daily weight gain is fast and lower during later infancy when daily weight gain slows down.
- Breast milk contains a multitude of bioactive proteins that are highly concentrated in early lactation and decrease with progressing lactation.
- Quantity and quality of protein in breast milk are crucial for healthy growth and long-term development.

Keywords
Protein requirements for growth · Nutritive proteins · Bioactive proteins · Bioactive peptides · Infants · Breastmilk · Lactoferrin · Immunoglobulins

Introduction
Breastfeeding is important for the healthy growth and development of infants and young children. The WHO recommends exclusive breastfeeding until 6 months and continuation of breastfeeding until 2 years as part of a mixed diet. However, recent DHS surveys indicate that even in developing countries only about 32% of mothers...
exclusively breastfeed their infants until 6 months [1], and the quality of complementary foods is very low. Therefore, in many developing countries, stunting is still prevalent in about 20% of children under 5 years of age [2]. In most developed countries, solids are introduced between 4 and 6 months, and breastfeeding is often stopped much earlier than recommended.

After carbohydrates and lipids, proteins are the third abundant solids in breast milk (BM), not only providing crucial amino acids indispensable for growth but also bioactive proteins and peptides essential for many functions (Table 1).

### Protein Needs for Growth

The protein intake of breastfed term infants has been used as a model to estimate protein requirements during the first year [3, 4]. The protein content in BM can be quantified by directly assessing the true protein content [5]. True protein concentrations of 14–16, 8–10, and 7–8 g/L have been reported during early lactation, at 3–4 months, and at 6 months, respectively. A recent meta-analysis of 43 studies [5] confirms that the protein concentration in BM depends on the stage of lactation and time since delivery. It also indicates a big variety in protein concentration, in particular during the first few months of lactation. However, the true protein intake does not accurately reflect the amount of utilizable amino acids to synthesize new body protein because some (bioactive) BM proteins can be found intact in infant stools [6].

Protein requirements for growth and daily turnover strongly depend on rates of body protein gain. Daily protein retention in the body can be calculated by measuring absorption and excretion. Fomon et al. [7] published detailed estimates of protein gain in children during the first 2 years and beyond. Based on total body water (estimating the extra- and intracellular compartment), total body potassium (estimating the intracellular compartment where almost all of the protein is present), and total body calcium (osseous minerals), all components of fat-free mass were calculated. Because potassium is the main intracellular cation, gains at different age ranges allow to estimate gain of total body protein (Fig. 1). Daily protein gain is highest in the very young infant and is rapidly decreasing during later infancy and the second year of life; during the first months, protein gain is 3 times higher than between 12 and 24 months. Indeed, protein concentration in BM is high during the first few weeks of lactation and then continues to decrease throughout the first year, but at substantially lower rates than those observed in the first weeks (Fig. 2). Casein and most whey proteins in BM are utilized for growth. Their concentrations change profoundly over the course of lactation: during the first 2 weeks of lactation, concentrations of whey proteins are

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**Table 1. Bioactive functions of breast milk proteins**

<table>
<thead>
<tr>
<th>Function</th>
<th>Bioactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune modulation and antimicrobial activity</td>
<td>Lactoferrin</td>
<td>29, 30, 113</td>
</tr>
<tr>
<td></td>
<td>Secretory IgA</td>
<td>36, 114</td>
</tr>
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<td></td>
<td>Osteopontin</td>
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<td></td>
<td>Cytokines</td>
<td>53, 54</td>
</tr>
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<td></td>
<td>Lysozyme</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>κ-Casein</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Lactoperoxidase</td>
<td>61, 62</td>
</tr>
<tr>
<td></td>
<td>Haptocorrin</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>α-Lactalbumin</td>
<td>70</td>
</tr>
<tr>
<td>Digestive function</td>
<td>Bile salt-stimulated lipase</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Amylase</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>α1-antitrypsin</td>
<td>86</td>
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<tr>
<td>Gut development</td>
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<td></td>
<td>Lactoferrin</td>
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<tr>
<td>Carriers for other nutrients</td>
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<td></td>
<td>Haptocorrin</td>
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<td>Folate-binding protein</td>
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<td>α-Lactalbumin</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>β-Casein</td>
<td>111, 112</td>
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**Fig. 1.** Daily protein gains of boys and girls aged 0–24 months. Gains are based on total body potassium measurements [7].
very high, while concentrations of caseins are low, which results in a whey:casein ratio as high as 80:20. The ratio drops to 65:35 by week 2 and stays constant at about 60:40 thereafter [5].

Proteins are polymer chains made of amino acids linked together by peptide bonds. During the digestion process, most proteins are decomposed to simple amino acids or small peptides which are absorbed. Amino acids which are absorbed and not oxidized are the building blocks of new protein which is synthesized in the body. Despite the reduction in protein over time, the nutritional value of protein in BM, as measured by the ratio of essential amino acids to total amino acids, appears to be consistent over time. These changes correlate well with the evolving needs of the growing infant [8].

Because of their higher daily protein gain per unit of body weight, low-birth-weight infants have higher protein requirements than term infants [9, 10]. Concentrations of protein and amino acids during the first weeks of lactation are higher in BM of mothers who deliver preterm than in BM of mothers who deliver at term [8]. However, feeding BM without supplements does not meet protein requirements, in particular those of very low- and extremely-low-birth-weight infants. Supplements which are on the market are based on protein fractions of cow’s milk or human donor milk. Supplementa-
tion of BM or the use of preterm formulas improves growth rates of low-birth-weight infants, but it is questionable if the right mix of indispensable amino acids for synthesis of new body protein in low-birth-weight infants has already been found.

Growth differences between breastfed infants and infants fed high-protein formulas (i.e. >2.25 g/100 kcal) have been shown more than 2 decades ago [11]. Infants fed a formula with high protein content grow faster during the first 2 years and beyond [12], and they have higher insulin and insulin-like growth factor 1 levels in blood [13, 14]. Rapid growth during the first year is associated with obesity during childhood. Three longitudinal studies confirm a strong relationship between weight gain between 0 and 12 months and BMI at 12, 36, and 60 months (Fig. 3) [10]: 21% of the variance of BMI at 60 months was explained by weight gain between birth and 12 months. Narrowing the protein gap between BM and infant formula requires a deep understanding of how protein quality and quantity in BM changes over time. Composition of infant formulas has evolved with increasing knowledge of BM. Recently, a pooled analysis of individual growth data (11 clinical trials; \( n = 1,882 \) [15]) of infants who received a modified whey-based low-protein starter formula (1.8 g protein/100 kcal) [16] with an amino acid profile close to term BM [17] has become available. The weight and length of formula-fed infants at 4 months correspond to the 50th percentile of the WHO global growth standard [18]. The CHOP study in Europe followed the growth of children who had been fed low- or high-protein formulas during the first year of life. At 6 years, BMI and the percentage of children with obesity were significantly lower in the low-protein formula group [12]. Two randomized controlled trials [19, 20] tested 2 low-protein follow-up formulas (1.6 g protein/100 kcal) and followed children until 5 years of age. BMI at 5 years of the children who had been on the low-protein formula was similar to that of the children who had been exclusively breastfed until 4–6 months. [10].

Therefore, it seems that the quantity and quality of protein in BM are crucial for healthy growth and long-term development. Some nutritive proteins which are partly or well absorbed may also have biological functions. In addition, there are bioactive proteins in BM which are not absorbed. Specific functions of bioactive proteins and peptides which have been studied in detail can provide insights on why breastfed infants have lower morbidity and shorter infection periods [21] as well as different microbiota [22].
Bioactive (Functional) Proteins in BM

Immunomodulatory and Antimicrobial Activity

Lactoferrin

Lactoferrin, also known as lactotransferrin, was reported to be present in bovine milk in the late 1930s and quantified in BM in the early 1960s [23]. Originally described as “red protein from (bovine) milk,” it turned out to be a multifunctional globular glycoprotein [24, 25]. Lactoferrin content of BM decreases with progressing stages of lactation, found highest in colostrum at 5.5 g/L and between 1.5 and 3.0 g/L in mature milk depending on the stage of lactation [26]. It is generally accepted that lactoferrin resists digestion to some degree and therefore can be found intact in infant feces. However, early in life, a fraction of this protein may be taken up by the intestinal mucosa and the rest is digested to yield bioactive peptides [27]. Due to its high affinity towards ferric iron, it not only acts as a carrier of iron in BM, but also deprives harmful microbes of iron that is key for their growth. Additionally, due to its basic N-terminal domain that can interact with various microbial and host targets, lactoferrin not only has antimicrobial properties but also modulates the innate and adaptive immune responses. This is orchestrated by cytokines interleukin (IL)-4, IL-2, IL-12, and interferon-γ and results in its ability to act as an anti- or proinflammatory agent. It has been demonstrated that lactoferrin can compete with lipopolysaccharide for binding to CD14 and thereby preventing lipopolysaccharide-mediated proinflammatory series of events [28]. Although this molecule is resilient to digestion and can be found intact in fecal material, it is digested to some extent to form lactoferricin, a molecule that is able to inhibit Escherichia coli attachment to intestinal cells [29, 30]. Lactoferricin may not be the only lactoferrin related peptide to have antimicrobial activity, the role of Lf(1–11) and lactoferrampin has also emerged in the recent past [31]. In one study, Lf(1–11) was demonstrated to be active against gram-positive bacteria (Staphylococcus spp. and Streptococcus mitis) as well as gram-negative bacteria (Acinetobacter baumannii, Pseudomonas spp., Klebsiella spp., and E. coli) [32].

Fig. 3. Weight gain between birth and 12 months predicts BMI at 12, 36, and 60 months. Data from 3 longitudinal studies (Italy, USA, Chile) [10].
Breastmilk: Nutritive and Bioactive Proteins

may contain on average 2.0 g/L of sIgA, which is reduced to approximately 0.5 g/L in mature milk [33]. The absorptive fate of this protein is similar to that of lactoferrin in that it is partly excreted intact and partly digested to bioactive peptides [27]. This class of antibody has been documented to be abundantly present in intestinal mucosa of humans and other mammals and to protect the epithelium from toxic assaults. As a first line of defense, in inhibiting infectious incident, sIgA would block toxin adhering to intestinal epithelium. In mouse models exposed to Vibrio cholerae toxin, sIgA demonstrated a protective effect [34]. Another mechanism by which sIgA could block pathogens is by direct recognition of receptor-binding domains like reovirus type 1 Lang. When IgA knockout mice were challenged with reovirus, the orally gavaged IgA group of knockout mice was as effective as wild-type mice in clearing the infection [35]. sIgA may also have a direct effect on the virulence of the bacteria. For example, murine monoclonal IgA-binding Shigella flexneri suppressed activity of the bacterial type 3 secretion (T3S), necessary for S. flexneri to gain entry into intestinal epithelium [36]. Immune exclusion is often referred to as sIgA’s ability to prevent pathogen access to intestinal epithelium through a series of processes involving agglutination, entrapment in mucus, and clearance via peristaltic movements [37].

Osteopontin

Osteopontin is a multifunctional, heavily glycosylated and phosphorylated acidic protein with possible roles in immune activation, inhibition of ectopic calcification, cellular adhesion and migration, angiogenesis, and bone remodeling [38]. With high variability amongst populations and stages of lactation, the average concentration of osteopontin in BM is approximately 140 mg/L [39]. When compared to wild-type suckling mice, osteopontin knockout suckling mice suffered from prolonged periods and intense bouts of diarrhea upon rotavirus exposure [40]. A fine balance between T helper 1 (Th1) and T helper 2 (Th2) is required to alleviate an immune response. Osteopontin has been demonstrated to reduce Th1 expression and inhibit Th2 along with IL-10 [38]. Interestingly, breastfed infants but not formula-fed infants showed induction of Th1 response when immunized with measles, mumps and rubella [41]. This observation can presumably be tied to presence of osteopontin in BM of the breast fed group but not the formula fed group. Furthermore, through electrostatic interactions, osteopontin can form complexes with lactoferrin and thereby acts as a carrier for other immunomodulatory proteins to further enhance the immune competency of its consumers [42]. Recently, a randomized controlled trial was carried out where 2 groups of formula-fed infants and a group of breast-fed infants were recruited. The 2 formula-fed groups were fed a standard formula with 65 mg/L bovine-derived osteopontin or an experimental formula containing 130 mg/L bovine-derived osteopontin. Apart from comparable growth parameters, differences were observed in lowered levels of proinflammatory cytokine TNF-α and a significantly fewer number of days when the infants had fever [43].

Cytokines

The effect of cytokines to regulate inflammatory processes often associated with infection is usually like an orchestra, operating in network and produces a cascading effect. Cytokines are postulated to enhance proliferation of thymocytes, inhibit IL-2 production from T-cells and suppress IgE production [44–46]. The presence of several cytokines in BM has been demonstrated over years. These molecules include, but may not be limited to, IL-1β, IL-6, IL-8, IL-10, TNF-α, interferon-γ, transforming growth factor-β, and colony-stimulating factor [47–52]. Usually they are present at very low concentrations (picograms) and potentially may originate from epithelial mammary gland cells, or activated macrophages and other cells in BM [53]. The biological function of these agents on infants is to complement infants’ own source of cytokines that are produced at lower quantities due to immaturity of the immune system. Although cytokines are not as well studied as other immunomodulatory agents described in this section, it is postulated that these molecules balance Th1 and Th2 to impart immunity-related benefits [54].

Lysozyme

Lysozyme belongs to the whey class of protein fraction in BM and possesses bactericidal properties by affecting the cell wall of most of gram-positive and some gram-negative bacteria. Higher amounts of lysozyme have been observed in colostrum at approximately 0.36 g/L that is reduced slightly in mature milk to 0.30 g/L [55]. Attempts have also been made to produce recombinant human lysozyme and lactoferrin in dairy animals [56]. The mechanism is yet unclear, but lysozyme of BM origin also contains activity against HIV type 1 [57].

κ-Casein

κ-Casein belongs to the casein family of phosphoproteins that is involved in a number of physiological processes. With an average concentration of approximately...
1.25 g/L in colostrum and transition milk, it settles closer to 1 g/L in mature milk [58]. These glycosylated κ-casein of human origin as opposed to bovine origin inhibit the cell lineage-specific adhesion of Helicobacter pylori to human gastric surface mucous cells [59].

Lactoperoxidase
A member of the heme peroxidase family, lactoperoxidase is secreted by mammary glands and is persistently present during lactation. In human BM, lactoperoxidase is found at 1–1.5 units/mL during the first 6 months of lactation [60]. It is well accepted that this peroxidase catalyzes oxidation of thiocyanate from the saliva of infants to hypoiodotyrosine in the presence of small amounts of hydrogen peroxides already in the mouth of the baby. The formed hypoiodotyrosine may be responsible to exterminate gram-positive and gram-negative bacteria [61, 62].

Haptocorrin
Haptocorrin is a vitamin B₁₂-binding protein found in many body fluids including BM with a concentration range of approximately 5 μg/mL in colostrum to 3 μg/mL in mature milk [63]. Structurally, haptocorrin did not show much alternations after exposure to digestive enzymes and was able to inhibit the growth of E. coli in an in vitro system [64]. Further systematic study of exposure of haptocorrin to 34 commensal and pathogenic bacteria indicated suppression of only Bifidobacterium breve implicating its role that may be limited to certain strains and a blanket antimicrobial label might not be relevant for this protein warranting further studies [65].

α-Lactalbumin
A well-characterized and primary protein in BM, α-lactalbumin is made up of 123 amino acids and 4 disulfide bridges and accounts for 20–25% of total BM proteins [66, 67]. Since it is also a rich source of many indispensable amino acids, a fraction of the protein is digested well and the rest yields polypeptides that exert antimicrobial activities mostly against gram-positive bacteria and not gram-negative bacteria [68, 69]. Additionally, a folding variant of α-lactalbumin was also found to be bactericidal against an antibiotic resistant strain of Streptococcus pneumonia [70]. Not only for its antimicrobial benefits but also to mimic the BM closer for additional benefits, all efforts have been made to enrich BM substitute with α-lactalbumin [71].

**Digestive Functions**

**Bile Salt-Stimulated Lipase**
The major source of energy for breast-fed infants is the predominant form of lipid in BM, the triacylglycerols. Milk triacylglycerols are efficiently digested by complementary actions of gastric lipase, colipase-dependent pancreatic lipase, and bile salt-stimulated lipase (BSSL). While there are 2 sources of these enzymes, infants’ exocrine pancreas, the major source is maternal milk BSSL. In the early 1950s, it was first demonstrated by Freudenberg that mothers’ milk contains an inactive lipase that is stimulated when the chyme reaches duodenum and comes in contact with bile salts [72, 73]. BSSL was purified and characterized in the early 1980s [74], is demonstrated to have a broad substrate specificity [75–77] and to inactivate by pasteurization of BM [78]. Therefore, digestion and absorption of lipids is significantly lower when pasteurized donor milk is fed to preterm infants [79]. Recently in a randomized phase 3 study, recombinant human BSSL was added to infant formula to assess if it had any impact on growth velocity, presumably due to better lipid digestion and absorption. Interestingly, the benefits on growth were not observed in appropriate-for-gestational-age preterm infants but were present in small-for-gestational-age preterm infants [80].

**Amylase**
In absence of pancreatic amylase, BM amylose may catalyze hydrolysis of starch, glycogen and other related saccharides by cleavage of α-1,4 linkages to produce maltose, dextrins, and glucose. Activity of amylase varies from 1,000 to 5,000 units per liter of BM [81]. Colostrum is known to contain higher activities compared to transition or mature milk [82]. There is a further decrease of approximately 35% of the activity beyond the first trimester of breastfeeding [83]. Additionally, higher parity may also reduce amylose activity by half [83]. Preterm milk contains equal amounts of amylose activity as term milk [84]. Apart from its obvious digestion-aiding role, amylose may also act as antibacterial by attacking the polysaccharides of the bacterial cell wall [82].
α1-antitrypsin
Thy physiological role of protease inhibitors like α1-antitrypsin (A1AT) in BM is not completely understood. However, as generally accepted for other mammals, protease inhibitors may play a role in digestion and/or absorption of bioactive proteins present at relatively higher concentrations in colostrum. Indeed, McGilligan et al. [85] showed the highest presence of A1AT in colostrum (1.4–5.2 g/L) compared to the first 26 weeks (0.07 g/L) or 26–52 weeks of lactation (0.05 g/L). A1AT resists digestion in the enteral tract and can be found intact in feces of infants in significant quantities [86]. Efforts have been made to express human A1AT in transgenic sheep for potential human applications [87, 88].

Gut Development
Growth Factors
Growth factors, their concentrations in BM, and biological sources of growth factors have been described in the literature [89]. Potentially originating from epithelial and stromal cells as well as from macrophages of mammary glands, the growth factors are present at microgram-per-liter quantities in BM. Growth factors that are present in the intestinal lumen, such as epidermal growth factor and insulin-like growth factors 1 and 2, originate either from salivary glands of the infants or from mothers’ milk [90]. It still remains unclear how they are able to exert effects upon their receptors that are located on the basolateral side of the absorptive intestinal epithelial cells. Indeed, one suggestion is that the immature gut of the infant provides access of the ligands to the basolateral compartment. Preterm infants, whose gut is relatively underdeveloped compared to that of term infants, may have significantly higher concentrations of epidermal growth factor in milk secreted by their mothers [91].

Lactoferrin
Lactoferrin exposure to intestinal cell culture models shows increased proliferation and differentiation in a dose-dependent manner [92]. Additionally, it also has greater proliferation of intestinal crypt cells in a piglet model [93]. Indeed, it is plausible that rapid maturation of absorptive intestinal epithelia in presence of lactoferrin may contribute to the higher weight gain in infants fed BM substitute with lactoferrin compared to a control group without lactoferrin [94].

Carriers for Other Nutrients
Lactoferrin
Iron absorption in breastfed infant was reported to be more efficient than cow’s milk-based infant formula [95]. Certainly, this was later attributed to the presence of relatively high levels of lactoferrin in BM compared to bovine milk (approx. 1 mg/mL and 10 μg/mL, respectively) and the majority of the iron in milk is bound to lactoferrin [25, 26]. Furthermore, a receptor of lactoferrin was later identified that had greater affinity for human lactoferrin in contrast to bovine lactoferrin for iron absorption [96]. Efforts are underway to express recombinant human lactoferrin in rice, and a comparison of the stability and bioactivity has shown promising results for its potential use in BM substitutes [97].

Haptocorrin
Haptocorrin, also known as transcobalamin 1, perhaps a name derived from “transporter of cobalamin,” an alternative name for vitamin B12, is a binding protein found in BM [98]. In adults, vitamin B12 absorption is dependent on digestive juices, enzymes, binding protein secreted in stomach, intrinsic factors, and their receptors in the small intestine [99]. However, in infants, very low amounts of intrinsic factors have been found in fecal material, perhaps indicating that haptocorrin may have a larger role to play in the transport of vitamin B12 [100].

Folate-Binding Protein
Identified in the late 1960s, folate-binding protein (FBP) almost entirely binds all naturally occurring folate in BM as well as bovine milk [101, 102]. Since its discovery, it has been thought that FBP sequesters various forms of folate and ensures adequate supply to the neonate by also preventing the oxidation in the digestive tract [103, 104]. Solids of pooled human and bovine milk contained approximately 2,000 nmol/kg, whereas goat milk contains twice as much and freeze-drying or spray-drying of milk to powder retains practically all FBP [105, 106]. Since FBP is able to withstand digestion, it is plausible that permeable intestinal lining of the infant gut is able to take up the folate-FBP complex at least for weeks or even months postpartum until the tight junctions are formed [54].

α-Lactalbumin
Originally proposed to carry divalent cations like calcium and zinc [54], it did show higher absorption in infant rhesus monkeys [107]. However, research work in human infants did not show any signs of altered absorption of iron α-lactalbumin-enriched infant formula [108], warranting further studies for this biological benefit.
β-Casein
The total casein concentration increases during lactation and represents approximately 10–20% during earlier stages and 40–50% when lactation matures [109, 110]. In mature milk, β-casein may represent up to 25% or approximately 2.7 g/L in BM [58]. This protein is highly phosphorylated, which at least in preclinical model has shown to solubilize calcium and uptake by intestinal cells at least in part by forming casein phosphopeptides that may act as calcium ionophores or calcium carriers across the membrane [111, 112]. More research remains to be done to elucidate the role of casein phosphopeptides in enhanced uptake of other divalent cations like zinc and even iron.

Conclusion
Recent findings on nutritive and bioactive proteins in breastfeeding support the WHO recommendations that breastfeeding should be continued during the first year and beyond. Infant formula manufacturers should eliminate all high-protein formulas from the market. New formulas for infants should be low in protein, in particular follow-up formulas and growing-up milks. Protein quality in formulas (amino acid profiles) should be closer to that in BM. Before bioactive proteins are added to infant formulas, safety and efficacy tests must be provided by formula manufacturers.

Disclosure Statement
F.H. is a board member of the Nestlé Nutrition Institute, a nonprofit-making Swiss association which receives educational grants from Nestec S.A., Switzerland, and other sources. S.K.T. is an employee of Nestlé Research Center, Lausanne, Switzerland.

References
The average intake of human milk lipids in fully breastfed infants amounts to 21.4 g/day or a total of 3.9 kg between birth and 6 months of age.

Human Milk Lipids
by Berthold Koletzko

Key insights
The lipid components of human milk provide the infant with energy and essential micronutrients, and they also serve specific roles to support gastrointestinal function, lipid and lipoprotein metabolism, neurodevelopment, and immunity. There have been significant advances both in food technology, which enables the supply of new lipid preparations, and in lipidomic analyses, which offer insight into the biological effects of complex lipids in infancy. These will pave the way for improvements in the feeding of infants who cannot be breastfed.

Current knowledge
Human milk lipids provide a major portion of the total energy intake in infants (approximately half of the energy supply). The concentration of milk lipids varies greatly between individuals, during the day, and throughout the course of breastfeeding. The hindmilk contains a higher fat composition and a correspondingly larger mean size of the milk fat globule. The outer layer of the milk fat globule membrane (MFGM) consists of a bilayer of amphipathic lipids, primarily phosphatidylcholine, sphingomyelin, and cholesterol, as well as cerebrosides, gangliosides, and others. These components are highly bioactive.

Practical implications
The biological importance of MFGM is gaining increased attention after several clinical trials reported benefits of adding components of MFGM to infant formula. Current evidence supports the provision of omega-3 docosahexaenoic acid along with omega-6 arachidonic acid with infant formula. The recent revision of the European legislation that was implemented in 2016 stipulates that all infant and follow-on formula must contain between 20 and 50 mg omega-3 docosahexaenoic acid per 100 kcal. delete the text marked in yellow and replace by “without a minimum requirement of arachidonic acid”. This is a novel concept never clinically tested for suitability and safety of healthy infants from birth, and indications of possible adverse effects exist. Therefore, we recommend not to use such formula until conclusive data on their safety might become available in the future.

Recommended reading
Human Milk Lipids

Berthold Koletzko

Ludwig-Maximilians-Universität Munich, Division of Metabolism and Nutritional Medicine, Dr. von Hauner Children’s Hospital, University of Munich Medical Center, Munich, Germany

Key Messages

- Human milk lipids provide a major portion of the energy supply to breastfed infants as well as essential vitamins, polyunsaturated fatty acids, complex lipids, and bioactive components.
- Recent data evaluating the addition of preparations of complex lipids with or without milk fat globule membranes to vegetable oil-based infant formula show promising indications for potential improvements of infant development and reduction of infection risk.
- Analyses of gene-diet interaction following the concept of Mendelian randomization add to the evidence that the supply of long-chain polyunsaturated fatty acids in infancy is causally related to improving cognitive development and to reducing asthma risk at school age. Current evidence supports the provision of omega-3 docosahexaenoic acid along with omega-6 arachidonic acid with infant formula.
- Significant methodological progress both in food technology enabling the provision of new lipid preparations and in lipidomic analyses offers major opportunities to explore the biological effects of the supply of complex human milk lipids.

Abstract

Human milk lipids provide the infant with energy and essential vitamins, polyunsaturated fatty acids, and bioactive components. Adding complex lipids and milk fat globule membranes to vegetable oil-based infant formula has the potential to enhance infant development and reduce infections. Cholesterol provision with breastfeeding modulates infant sterol metabolism and may induce long-term benefits. Some 98–99% of milk lipids are comprised by triacylglycerols, whose properties depend on incorporated fatty acids. Attention has been devoted to the roles of the long-chain polyunsaturated fatty acids docosahexaenoic (DHA) and arachidonic (ARA) acids. Recent studies on gene-diet interaction (Mendelian randomization) show that breastfeeding providing DHA and ARA improves cognitive development and reduces asthma risk at school age particularly in those children with a genetically determined lower activity of DHA and ARA synthesis. It appears prudent to follow the biological model of human milk in the design of infant formula as far as feasible, unless conclusive evidence for the suitability and safety of other choices is available. The recent European Union legislative stipulation of a high formula DHA content without required ARA deviates from this concept, and such a novel formula composition has not been adequately evaluated. Great future opportunities arise with significant methodological progress for example in lipidomic analyses and their bioinformatic evaluation, which should enhance understanding of the biology of human milk lipids. Such knowledge might lead to improved dietary advice to lactating mothers as well as to further opportunities to enhance infant formula composition.

Keywords

Breastfeeding · Milk fat globule membranes · Phospholipids · Sphingomyelins · Gangliosides · Arachidonic acid · Docosahexaenoic acid

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Introduction

Lipids are a major source of energy provided with human milk to the infant [1, 2], but they also provide essential nutrients such as polyunsaturated fatty acids (PUFA) and lipid soluble vitamins. Many studies have demonstrated important biological effects of the milk lipids provided to the recipient infant, for example on gastrointestinal function, lipid and lipoprotein metabolism, membrane composition and function, infant growth, neurodevelopment, and immune function [3].

Human milk lipids provide a major portion of the total energy intake in young infants, with a mean 44% of the energy supply [4] (Fig. 1). The average intake of human milk lipids in fully breastfed infants amounts to 21.42 g/day between birth and 6 months of age [4]. This results in an impressive 3.9 kg of human lipid supplied during the first half year of life to fully breastfed infants, equivalent to some 35,000 kcal provided by human milk lipids alone during the first 6 months of life. While the mean lipid content in human milk is relatively stable during the course of the first months of lactation, there is very wide interindividual and intraindividual variation of milk fat concentrations (Table 1) [4–6]. In fact, among the macronutrients in milk, fat shows the most variable concentration. For example, in mature milk samples collected at the infant age of 2 months, we find a coefficient of variation of 37.3% for milk fat but only of 14.4% for lactose and 12.9% for protein [4]. Milk fat content tends to increase

Table 1. Longitudinal evolution of human milk constituents in 30 prospectively followed lactating women

<table>
<thead>
<tr>
<th>Age</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
<th>6 months</th>
<th>Intraclass correlation coefficient²</th>
<th>Change in mean over time, p value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal/100 mL</td>
<td>66.1 (11.1)</td>
<td>68.3 (13.4)</td>
<td>63.0 (10.5)</td>
<td>62.4 (13.3)</td>
<td>0.40</td>
<td>0.065*</td>
</tr>
<tr>
<td>Carbohydrates, g/L</td>
<td>7.28 (1.36)</td>
<td>8.05 (1.15)</td>
<td>7.84 (1.39)</td>
<td>7.96 (1.74)</td>
<td>0.04</td>
<td>0.135</td>
</tr>
<tr>
<td>Lactose, g/L</td>
<td>72.4 (13.5)</td>
<td>80.3 (11.6)</td>
<td>78.0 (13.9)</td>
<td>79.2 (17.3)</td>
<td>0.04</td>
<td>0.129</td>
</tr>
<tr>
<td>Galactose, g/L</td>
<td>0.13 (0.04)</td>
<td>0.11 (0.03)</td>
<td>0.11 (0.04)</td>
<td>0.09 (0.03)</td>
<td>0.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protein, g/100 mL</td>
<td>1.38 (0.16)</td>
<td>1.16 (0.15)</td>
<td>1.04 (0.13)</td>
<td>0.96 (0.16)</td>
<td>0.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-protein nitrogen, g/dL</td>
<td>0.23 (0.02)</td>
<td>0.20 (0.02)</td>
<td>0.18 (0.02)</td>
<td>0.17 (0.02)</td>
<td>0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat, g/100 mL</td>
<td>3.20 (1.27)</td>
<td>3.16 (1.18)</td>
<td>2.92 (1.23)</td>
<td>2.71 (1.25)</td>
<td>0.40</td>
<td>0.164</td>
</tr>
<tr>
<td>Saturated fatty acids¹</td>
<td>39.0 (5.62)</td>
<td>37.7 (4.38)</td>
<td>37.2 (4.82)</td>
<td>36.8 (4.64)</td>
<td>0.21</td>
<td>0.202</td>
</tr>
<tr>
<td>Monounsaturated fatty acids¹</td>
<td>45.8 (4.62)</td>
<td>46.7 (4.48)</td>
<td>47.0 (4.25)</td>
<td>47.0 (4.26)</td>
<td>0.31</td>
<td>0.517</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (PUFA)¹</td>
<td>15.2 (4.26)</td>
<td>15.6 (2.95)</td>
<td>15.7 (3.43)</td>
<td>16.3 (4.17)</td>
<td>0.38</td>
<td>0.530</td>
</tr>
<tr>
<td>18:2n-6 (linoleic acid)¹</td>
<td>12.8 (3.88)</td>
<td>13.2 (2.81)</td>
<td>13.5 (3.32)</td>
<td>14.0 (4.08)</td>
<td>0.41</td>
<td>0.435</td>
</tr>
<tr>
<td>20:4n-6 (arachidonic acid)¹</td>
<td>0.51 (0.16)</td>
<td>0.52 (0.13)</td>
<td>0.52 (0.10)</td>
<td>0.52 (0.15)</td>
<td>0.31</td>
<td>0.981</td>
</tr>
<tr>
<td>18:3n-3 (α-linolenic acid)¹</td>
<td>0.62 (0.16)</td>
<td>0.69 (0.18)</td>
<td>0.61 (0.14)</td>
<td>0.67 (0.13)</td>
<td>0.16</td>
<td>0.074</td>
</tr>
<tr>
<td>20:5n-3 (EPA)¹</td>
<td>0.12 (0.03)</td>
<td>0.12 (0.03)</td>
<td>0.10 (0.03)</td>
<td>0.12 (0.05)</td>
<td>0.31</td>
<td>0.090</td>
</tr>
<tr>
<td>22:6n-3 (DHA)¹</td>
<td>0.25 (0.11)</td>
<td>0.24 (0.11)</td>
<td>0.26 (0.09)</td>
<td>0.30 (0.15)</td>
<td>0.21</td>
<td>0.206</td>
</tr>
<tr>
<td>n-3 LC-PUFA¹</td>
<td>0.48 (0.15)</td>
<td>0.48 (0.16)</td>
<td>0.49 (0.13)</td>
<td>0.56 (0.23)</td>
<td>0.17</td>
<td>0.148</td>
</tr>
<tr>
<td>n-6 LC-PUFA¹</td>
<td>1.22 (0.34)</td>
<td>1.22 (0.30)</td>
<td>1.17 (0.20)</td>
<td>1.11 (0.31)</td>
<td>0.34</td>
<td>0.229</td>
</tr>
</tbody>
</table>

Values are mean and SD. The intraclass correlation coefficient that reflects the stability of human milk constituents over time in each woman indicates a very high intraindividual variation for carbohydrates, while stability over time was higher for milk energy, protein, and fat content. Among fatty acids, omega-3 fatty acids had the lowest intraclass correlation coefficient. ¹ % fatty acid of milk total lipids. ² Based on linear random-effects model with subject as a random effect and month as fixed effect. * Linear trend.

Modified from Grote et al. [4].
Milk can be characterized as an emulsion of milk fat globules in an aqueous liquid. Milk fat globules with markedly variable sizes are formed in the mammary alveolar cells and contain a core of nonpolar lipids comprised primarily of triacylglycerols, with added small amounts of monoglycerides, diglycerides, and nonesterified fatty acids. These nonpolar lipids are formed in the endoplasmic reticulum from fatty acids obtained from the maternal circulation as well as primarily intermediate-chain fatty acids with 12 and 14 carbon atoms synthesized from acetyl-CoA. Upon the secretion from the endoplasmic reticulum of mammary epithelial cells into the cytosol, this triglyceride-rich core is covered by an inner membrane derived from the endoplasmic reticulum consisting of a monolayer primarily of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cholesterol. When these lipid droplets are further excreted from mammary epithelial cells into the alveolar space, they are covered by a piece of the apical plasma membrane, which results in the addition of another phospholipid bilayer and the other components of the mammary epithelial cell membrane such as membrane proteins and glycoproteins (Fig. 3). This outer layer of the milk fat globule membrane (MFGM) consists of a bilayer of amphipathic lipids, primarily phosphatidylcholine, sphingomyelin, and cholesterol, as well cerebrosides, gangliosides, glycosylated proteins and polypeptides, filaments, mucins, lactadherin, butyrophilin, and others; hence, MFGM contain a high density of bioactive components [9].

Phospholipids, plasmalogens, and sphingolipids including ceramides and gangliosides provide about 0.2–1% of total milk lipids or about 100–400 mg/L [2]. The concentration of different phospholipids per 100 g milk were reported as 8.5 mg sphingomyelin, 6.8 mg phosphatidylethanolamine, 6.0 mg phosphatidylcholine, 1.4 mg phosphatidylserine, and 1.1 mg/100 g for phosphatidylinositol [10]. Phospholipids serve structural roles as indispensable components of all plasma membranes of body cells and organelles, and they have an impact on membrane functions and metabolism. Complex lipids also have roles in signal transmission and cell recognition [2, 3]. Gangliosides contribute some 10% of brain lipids, with high concentrations in the cerebral cortex.

The biological importance of MFGM is getting increased attention after several controlled trials reported benefits of adding bovine MFGM of complex lipid fractions to infant formula with fat derived predominantly from vegetable oil. A trial on formula enriched with sphingomyelin in preterm infants reported neurobehavioral benefits [11]. In a small trial in Indonesia, the addition of a ganglioside-rich bovine milk lipid fraction was reported to improve the hand and eye coordination IQ, performance IQ, and total IQ assessed with the Griffiths Mental Developmental Scale at the age of 24 weeks [12].

Fig. 2. Milk fat concentration in fore- and hindmilk collected before and after breastfeeding of 15 term infants. Drawn from data of Khan et al. [79].
Another trial providing a milk formula with addition of a similar preparation for 12 weeks enrolled 450 infants aged 8–24 months in India and reported no difference for rotavirus or for all-cause diarrhea. In a large study that enrolled more than 500 Peruvian infants, MFGM-supplemented formula did not affect diarrhea incidence but reduced longitudinal diarrhea prevalence [13]. A larger trial that enrolled more than 250 toddlers aged 2.5–6 years in Belgium reported that a milk preparation enriched with a phospholipid-rich lipid fraction resulted in less days with fewer and lower parental scoring of internal, external, and total behavioral problems [14]. A further trial enrolled 160 formula-fed infants in Sweden as well as a breastfed reference group and evaluated effects of added bovine MFGM, along with reduced formula contents of energy and protein. The MFGM group achieved higher cognition scores in the Bayley test at the age of 1 year (Fig. 4) and showed a much lower incidence of acute otitis media as well as less use of antipyretic drugs [15, 16]. These observations lead to the conclusion that MFGM and/or the complex lipids provided with the MFGM fraction may have important biological roles for the development of nervous and immune functions.

**Cholesterol**

Milk fat globule lipids also provide considerable amounts of free and esterified cholesterol, resulting in a total cholesterol content of 90–150 mg/L in human milk in contrast to typically only 0–4 mg/L in infant formula. Cholesterol is an indispensable building block for all cell membranes and is incorporated in considerable amounts...
into myelin in the nervous system during the period of rapid brain growth, and it serves as the substrate for the synthesis of bile acids, lipoproteins, vitamin D, hormones, and oxysterols that modulate cholesterol, lipid, and glucose homeostasis [3, 9, 17–19]. The provision of cholesterol with breastfeeding is associated with higher plasma concentrations of total and low-density lipoprotein cholesterol in breastfed than in formula-fed infants [20]. The provision of preformed cholesterol is most likely the cause for the about 3-fold lower endogenous cholesterol synthesis rate in breastfed than formula-fed infants, since the synthesis rate is inversely correlated to the daily cholesterol supply in mg/kg bodyweight [21]. In formula-fed piglets, dietary cholesterol supply downregulated hepatic hydroxymethylglutaryl coenzyme A reductase, the rate regulating enzyme for endogenous cholesterol synthesis [22]. In human infants aged 4 months, the rate of endogenous cholesterol synthesis also appeared to be regulated by dietary cholesterol supply. Breastfed infants with a high cholesterol and low phytoestrogen intake had the lowest fractional synthesis rate, while infants receiving cows’ milk-based formula with low cholesterol and low phytoestrogen had an intermediate rate, and infants fed soy-based formula with no cholesterol and high phytoestrogen had the highest rate of synthesis [23]. When cholesterol was added to the soy-based infant formula, the rate of synthesis was changed to similar results as in infants fed cows’ milk-based formula, which leads to the conclusion that the amount of dietary cholesterol supply regulates cholesterol synthesis in infants. Lasting effects of early feeding on later cholesterol levels were reported in several studies and reviewed in meta-analyses. A rather modest lowering of total and low-density lipoprotein cholesterol was found in adults who had been breastfed in infancy, compared to previously formula-fed people, with a greater effect size of exclusive than of partial breastfeeding [24, 25]. It was proposed that if 30% of infants are exclusively breastfed, resulting in a blood cholesterol reduction in adulthood by 0.15 mmol/L, the population prevalence of cardiovascular disease could be reduced by as much as 5% [25]. However, Ip et al. [26] noted that the analysis reporting reduced serum lipid levels in previously breastfed adults did not segregate the data according to gender and did not explicitly analyze potential confounders; they concluded that in view of the limited methodological quality of the meta-analysis the relationship between breastfeeding and adult cholesterol levels cannot be correctly characterized. Meta-analyses of available data do not allow definitive conclusions regarding the relationship between breastfeeding and all-cause mortality from cardiovascular diseases in adult life, although the confidence limits around the point estimates and the observed between-study heterogeneity do not exclude potential beneficial or adverse cardiovascular effects of breastfeeding [26, 27]. Therefore, it appears particularly promising to evaluate the short- and long-term effects of addition of well bioavailable preparations of cholesterol to infant formula in randomized controlled trials, which may shed further light on the potential biological importance of a dietary cholesterol supply in infancy.

**Fatty Acids Provided with Milk Lipids**

Triacylglycerols contribute some 98–99% of human milk fat. The properties of milk triglycerides are very much influenced by their fatty acid composition. Milk lipids of European women today typically contain some 35–40% saturated fatty acids, 45–50% monounsaturated fatty acids, and approximately 15% PUFA (Table 2). The saturated palmitic acid (C16:0) provides approximately 25% of all milk fatty acids and hence the major part of the total saturated fatty acid content. About 70% of human milk palmitic acid is esterified in the middle position (sn-2 position) of triacylglycerols which facilitates absorption. During intestinal digestion, fatty acids in the sn-1 and sn-2 positions are liberated as nonesterified fatty acids by pancreatic lipases. These nonesterified fatty acids are quite well absorbed if they are unsaturated and hence better water soluble. In contrast, liberated long-chain saturated fatty acids, such as palmitic acid, are poorly water soluble and poorly absorbed, but rather bind to calcium and form calcium soaps that are excreted with stools, thereby reducing both fat and calcium absorption. However, if palmitic acid is esterified in the sn-2 position, as it is predominantly the case in human milk lipids, pancreatic lipolysis yields a palmitoyl-monoglycerol which is well water soluble and well absorbed, thereby reducing fat and calcium malabsorption [28].

The human milk contents of the mono-unsaturated fatty acid oleic acid (C18:1n-9) and of the essential PUFA linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) vary with the maternal dietary intake of these fatty acids. This is illustrated by the approximately 3-fold increase of linoleic acid content in mature human milk in the USA since the mid 1940s, along with the increase of dietary vegetable oil and linoleic acid consumption in the population, whereas α-linolenic acid contents have remained rather constant (Fig. 5) [29]. Thereby the average ratio of the omega-6 linoleic acid to the omega-3 α-linolenic acid in human milk has also increased approximately 3-fold. We
studied the transfer of linoleic acid provided to lactating women into their milk using stable isotope-labelled fatty acids. An oral dose of 1 mg/kg bodyweight of linoleic acid uniformly labelled with the stable carbon isotope $^{13}$C was provided repeatedly during the 2nd, 6th, and 12th week of lactation [30]. Before and at several times during a 5-day period after tracer intake, samples of breath and milk were collected, the volume of daily milk production was assessed, and dietary nutrient intakes were calculated from prospective dietary protocols. Some 3.5–4.5% of the ingested linoleic acid was oxidized to CO$_2$ and exhaled with breath, with no significant differences between the studied time points. Dietary linoleic acid was rapidly transferred into milk, with a peak enrichment reached about 12 h after intake (Fig. 6). Linoleic transfer into milk in unchanged form or as its metabolites did not change during the course of lactation. The data indicate that about 30% of milk linoleic acid is derived directly from dietary intake, whereas about 70% originates from maternal body fat stores. It is tempting to speculate that this largely indirect transfer of dietary linoleic via intermediate body stores may represent a biological benefit to the breastfed infant, since this mechanism buffers short-term variation of maternal dietary supply of the parent essential fatty acid and provides the infant with a relatively stable parent essential fatty acid supply. However, long-term changes in dietary supply will also modify maternal body fat stores and thereby explain the observed marked changes over time (Fig. 5). Only about 11% of the milk content of the linoleic acid metabolite dihomo-$\gamma$-linolenic acid (C20:3n-6) in milk originates from direct endogenous conversion of maternal dietary linoleic acid, while only 1.2% of the milk arachidonic acid (ARA, C20:4n-6) is directly derived from maternal linoleic acid intake [30].

### Table 2. Absolute fatty acid supply with human milk in prospectively followed lactating women

<table>
<thead>
<tr>
<th>Age</th>
<th>1 months</th>
<th>2 months</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids</td>
<td>7,420.3 (2,425.5)</td>
<td>7,911.4 (2,398.4)</td>
<td>7,344.1 (2,390.0)</td>
<td>4,205.1 (3,107.4)</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td>8,712.8 (2,998.6)</td>
<td>9,821.8 (3,115.3)</td>
<td>9,238.6 (2,974.8)</td>
<td>5,344.3 (3,953.1)</td>
</tr>
<tr>
<td>PUFAs</td>
<td>2,851.5 (913.8)</td>
<td>3,278.8 (1,063.0)</td>
<td>3,082.1 (999.4)</td>
<td>1,884.8 (1,454.4)</td>
</tr>
<tr>
<td>18:2n-6 (linoleic acid)</td>
<td>2,407.0 (767.2)</td>
<td>2,764.9 (915.0)</td>
<td>2,635.1 (859.7)</td>
<td>1,619.5 (1,275.4)</td>
</tr>
<tr>
<td>20:4n-6 (arachidonic acid)</td>
<td>95.6 (32.9)</td>
<td>109.6 (38.6)</td>
<td>101.1 (33.1)</td>
<td>58.7 (43.5)</td>
</tr>
<tr>
<td>18:3n-3 (α-linolenic acid)</td>
<td>118.8 (47.7)</td>
<td>144.7 (49.0)</td>
<td>118.8 (39.1)</td>
<td>76.8 (58.2)</td>
</tr>
<tr>
<td>20:5n-3 (EPA)</td>
<td>22.7 (9.23)</td>
<td>24.2 (7.90)</td>
<td>20.4 (6.45)</td>
<td>14.1 (10.77)</td>
</tr>
<tr>
<td>n-3 LC-PUFAs</td>
<td>48.5 (25.5)</td>
<td>51.3 (20.2)</td>
<td>50.3 (17.1)</td>
<td>32.7 (23.4)</td>
</tr>
<tr>
<td>n-6 LC-PUFAs</td>
<td>228.7 (75.4)</td>
<td>256.9 (86.5)</td>
<td>229.7 (72.7)</td>
<td>126.3 (92.2)</td>
</tr>
<tr>
<td>n-3 PUFAs</td>
<td>215.9 (85.2)</td>
<td>244.1 (81.6)</td>
<td>209.6 (66.1)</td>
<td>138.9 (99.5)</td>
</tr>
<tr>
<td>n-6 PUFAs</td>
<td>2,635.7 (836.0)</td>
<td>3,021.8 (990.9)</td>
<td>2,865.0 (927.9)</td>
<td>1,745.8 (1,362.9)</td>
</tr>
</tbody>
</table>

Values are mean mg/day (SD). PUFAs, polyunsaturated fatty acids. Modified from Grote et al. [4].
Long-Chain Polyunsaturated Fatty Acids

The provision of LC-PUFA with milk, in particular of omega-6 ARA and omega-3 docosahexaenoic acid (DHA), has received considerable attention, because many of the biological effects of the essential fatty acids in early life appear to be mediated by LC-PUFA rather than the precursor essential fatty acids. Brenna et al. [31] performed a systematic review on 106 studies of human breast milk worldwide and culled to include only studies that used modern analysis methods capable of making accurate estimates of fatty acid contents as well as criteria related to the completeness of reporting. The final analysis included 65 studies with milk of 2,474 women. The authors found a milk ARA content of 0.47 ± 0.13% (mean ± SD, % wt/wt), whereas milk DHA content was lower at 0.32 ± 0.22% [31]. Higher milk DHA contents were found in coastal populations and those with regular marine food consumption. The greater stability of milk ARA levels with a coefficient of variation of only 29%, as compared to DHA with a coefficient of variation of 69%, appears to reflect a greater degree of metabolic regulation of milk ARA content. Stable isotope studies have led us to the conclusion that 90% of human milk ARA are not derived directly from absorbed dietary lipids but rather from maternal ARA body stores [32]. In contrast, dietary DHA supply is a key determinant of milk DHA content. We showed that the dietary DHA intake is linearly correlated to milk DHA [33] (Fig. 6). Breastfeeding women need to achieve a daily DHA intake of at least 200 mg to provide milk with a DHA content of at least 0.3%, which is required for a fully breastfed infant to obtain the daily supply of about 100 mg DHA/day considered desirable to meet metabolic needs [34]. Given that the regulation of human milk ARA and DHA content differs, milk DHA and ARA are not closely correlated (r = 0.25, p = 0.02) [31], and the ARA/DHA ratio is not constant. It remains controversial whether the ratio of ARA to DHA in milk – or rather the amounts of DHA and of ARA supplied – are of greater relevance for biological effects in the infant. A balanced supply of both ARA and DHA appears to be relevant for the adequate incorporation of ARA and DHA into the growing brain [35].

In view of the marked accretion of ARA and DHA in the growing brain and the ample experimental evidence of the impact of LC-PUFA on membrane function, eicosanoid and docosanoid formation and the resulting regulation of physiological processes as well as the development and function of neural and immune tissues, the impact of LC-PUFA provision with human milk and also with infant formula has received considerable interest.

The provision of DHA was shown to enhance the early development of visual acuity. The European Food Safety Authority (EFSA) concluded that a cause and effect relationship has been established between the intake of infant and follow-on formula supplemented with DHA at levels around 0.3% of total fatty acids and visual function at 12 months in formula-fed infants born at term from birth up to 12 months and in breastfed infants after weaning up to 12 months [36]. However, some controversy remains with regards to the effects of the supply of preformed LC-PUFA on neurodevelopment of healthy term infants. For example, the authors of a meta-analysis on randomized trials evaluating infant formula with LC-PUFA compared to formula without LC-PUFA concluded that while some studies showed a significant benefit, overall no significant effect was detectable [37, 38]. The authors noted the limitation of their conclusions by a large degree of heterogeneity of the included studies, which provided markedly different interventions and also used a variety of very different outcomes and approaches to outcome assessment. Of importance, the included studies did not adjust for the major impact of genetic variation modulating the rate of endogenous synthesis of LC-PUFA and related clinical endpoints, in particular variation in the Fatty Acid Desaturase (FADS) gene cluster [39, 40]. The lack of adjusting for this major modulating confounding factor in the included studies may considerably reduce the sensitivity to detect effects.
of dietary LC-PUFA effects. The comparison of breastfed infants provided with preformed LC-PUFA with infants fed formula without LC-PUFA in observational studies is also difficult to interpret, because human milk LC-PUFA and particularly DHA supply are closely associated with different dietary and lifestyle choices, including maternal smoking and parental socioeconomic status, which may also influence neurodevelopmental outcomes.

Further insight into PUFA effects are offered by considering the interaction of breastfeeding, which always supplies preformed LC-PUFA, and the genetic variation in the FADS gene cluster that predicts the enzyme activities of fatty acid desaturases 1 and 2. Gene variants of the FADS gene cluster have a major impact on the fatty acid composition of blood, tissues, and human milk [39–41]. We assessed the single-nucleotide polymorphisms in the FADS genes along with human milk fatty acid composition in 772 breastfeeding mothers who participated in the prospective Ulm Birth Cohort study both at 1.5 months after infant birth and at 6 months postpartum in a subset of 463 mothers who were still breastfeeding at this time [42]. At both time points, we found significant associations of FADS genotype with milk ARA contents and the ratio of ARA to dihomo-γ-linolenic acid, indicating that maternal FADS genotypes have an impact on the formation of LC-PUFA provided with breastmilk [42]. The variation of FADS genotypes was shown to also modulate the interaction of breastfeeding and cognitive development. Genotyping for the rs174575 variant in the FADS2 gene was performed in 5,934 children participating in the ALSPAC study in whom IQ tests had been performed at the age of about 8 years [43]. In line with other observational studies, previously breastfed children had higher IQ scores than previously formula fed children, but the relative impact of human milk nutrient supply and of confounding factors associated with breastfeeding cannot be easily deciphered from these observational data alone. Causal inferences on the role of human milk LC-PUFA supply can be drawn from the fact that the beneficial effect of breastfeeding was much more pronounced, with an added advantage of about 4.5 IQ points, in the group of children with a genotype predicting a low ability of LC-PUFA synthesis [43]. Replication of these findings was published with the analysis of data from 2 Spanish birth cohort studies [44]. Since the genotype is considered to be distributed in the population at random (“Mendelian randomization”) and unrelated to the parental decision to breastfeed and to other related lifestyle predictors of IQ at school age, these data provide powerful evidence for the causality between early LC-PUFA supply and status during the breastfeeding period and later IQ achievements.

The relevance of LC-PUFA supply for child neurodevelopment was also demonstrated in a randomized clinical trial that enrolled 119 breastfeeding women in Texas, USA [45]. The women were assigned to receive identical capsules containing either a high-DHA algal oil providing approximately 200 mg DHA daily or a vegetable oil without DHA from delivery until 4 months after birth. Provision of DHA to the mother increased DHA in milk by about 70%, and in infant plasma phospholipids by about 20% [45]. At the age of 30 months, child psychomotor development was significantly better if mothers had received added DHA during the first 4 months of breastfeeding. At the age of 5 years, there were no differences in visual function, but children whose mothers had received added DHA performed significantly better on the Sustained Attention Subscale of the Leiter International Performance Scale (46.5 ± 8.9 vs. 41.9 ± 9.3, p < 0.008). These results support the conclusion that the DHA supply during early infancy is of importance for specific aspects of neurodevelopment.

Mendelian randomization also provided strong support for the conclusion that the LC-PUFA supply with breastfeeding is causally linked to protection against a later manifestation of bronchial asthma. Many studies have reported a protective effect of breastfeeding on asthma development, even though results are not consistent [26]. We evaluated the influence of the FADS1 FADS2 gene cluster polymorphisms on the association between breastfeeding and asthma in 2,245 children participating in 2 prospective German birth cohort studies, the GINI and LISA studies [46]. Logistic regression modelling was used to analyze the association between exclusive breastfeeding and doctor-diagnosed asthma occurring up to the age of 10 years, stratified by genotype. In the stratified analyses, heterozygous and homozygous carriers of the minor allele that show a low activity of LC-PUFA synthesis had a much reduced risk for later asthma if they were breastfed for 3 or 4 months and hence were provided with preformed LC-PUFA that can compensate for low endogenous synthesis (adjusted odds ratio between 0.37 [95% CI: 0.18–0.80] and 0.42 [95% CI: 0.20–0.88]). Interaction terms of breastfeeding with genotype were significant and ranged from $-1.17 \ (p = 0.015)$ to $-1.33 \ (p = 0.0066)$. Similarly, heterozygous and homozygous carriers of the minor allele who were exclusively breastfed for 5 or 6 months after birth had a reduced risk of asthma (0.32 [0.18–0.57] to 0.47 [0.27–0.81]) in the stratified analyses. In contrast, in individuals carrying the homozygous major allele pre-
dicting a greater degree of endogenous LC-PUFA formation, breastfeeding with provision of LC-PUFA showed no significant effect on asthma development. These results of a Mendelian randomization study demonstrate a lasting causal protection of breastfeeding for at least 3 months against doctor-diagnosed asthma until school age in children with a low rate of LC-PUFA synthesis and a modulating effect of postnatal PUFA status.

A systematic review on human studies on roles of LC-PUFA and an expert workshop that reviewed the information and developed recommendations was recently performed with support from the Early Nutrition Academy [34]. It was concluded that breastfeeding women should get ≥200 mg DHA/day to achieve a human milk DHA content of at least ≥0.3% of fatty acids. Infant formula for term infants should contain DHA and ARA to provide 100 mg DHA/day and 140 mg ARA/day, and a supply of 100 mg DHA/day should continue during the second half of infancy. No quantitative advice on ARA levels in follow-on formula fed after the introduction of complimentary feeding was provided due to lack of sufficient data and considerable variation in ARA amounts provided with complimentary foods.

**The advice to provide infant formula from birth that supplies DHA but no ARA has been heavily criticized**

**Should Infant Formula LC-PUFA Composition Be Guided by Human Milk Composition?**

With regards to infant and follow-on formula, the recent revision of the European legislation that came into force in 2016 stipulates that all infant and follow-on formula must contain between 20 and 50 mg DHA/100 kcal (approx. 0.5–1% of fatty acids), whereas formula without DHA content will not be allowed any more to be placed on the European Union market once this legislation is implemented [47]. To the great surprise of many pediatricians and of experts in the field, no requirement for a minimum content of ARA in infant formula has been defined. This legal regulation is based on advice provided by the European Food Safety Authority that reviewed a variety of aspects and nutrients, including LC-PUFA DHA and ARA. In a first report on nutrient requirements and dietary intakes of infants and young children published in 2013, adequate LC-PUFA intakes were defined as 100 mg DHA/day and 140 mg ARA/day from birth to the age of 6 months, while 100 mg DHA/day were considered adequate from 6 to 24 months [48]. These conclusions are in line with many other scientific reports, including the recent recommendations of the Early Nutrition Academy-supported global expert group that are based on a systematic review of the available scientific evidence [34]. In contrast, the subsequently published EFSA report on the compositional requirements of infant and follow-on formula advised that all infant and follow-on formula should contain relatively high amounts of DHA at 20–50 mg/100 kcal, but without the need to provide any preformed ARA [49]. This DHA level stipulated by EFSA and the new European legislation is much higher than the about 0.2–0.3% DHA found in most LC-PUFA-enriched formulae for term infants currently marketed around the world, which, however, generally also contain preformed ARA at levels equal to or often 2-fold higher than the DHA content. The proposed obligatory inclusion of DHA in all infant and follow-on formulae is welcomed by many scientists and pediatricians in view of the indications for beneficial effects [34], but the advice to provide infant formula from birth that supplies DHA but no ARA has been heavily criticized [50]. During pregnancy and infancy, both DHA and ARA are deposited in relatively large amounts in human tissues, including the brain [51, 52]. Fetal accretion of both DHA and ARA during pregnancy is facilitated by their active and preferential maternofetal placental transfer [53]. Pregnant women’s red blood cell levels of both DHA and ARA were positively associated with their children’s IQ at school age [54]. At birth, higher cord blood contents of both DHA and ARA predicted less later behavioral problems, emotional difficulties, hyperactivity, and attention deficit at the age of 10 years [55]. After birth, breastfed infants always get both preformed DHA and ARA, usually with a higher provision of ARA than of DHA [31, 56]. DHA along with ARA have been added to infant formulae since the 1980s in an attempt to approach the nutrient supply and functional effects achieved with breastfeeding [57–59]. The global Codex Alimentarius standard on the compositional requirements for infant formula stipulates the optional addition of DHA to infant formula, provided that the ARA content is equal to or higher than the DHA content, thus following the model of typical human milk composition [60]. Infant formulae providing both DHA and ARA have been evaluated in many controlled trials in infants [34]. In contrast, the proposed composition of term infant formula with up to 1% DHA and no ARA is a novel approach that has not been systematically tested for its suitability.
and safety in healthy infants born at term. ARA is an essential component of all cell membranes. The amount of ARA incorporated into the developing brain during infancy exceeds the deposition of DHA. Although humans can synthesize ARA to some extent from linoleic acid, infants fed formula without preformed ARA tend to develop lower ARA levels in blood plasma and erythrocytes than breastfed infants who receive both DHA and ARA [51, 57, 61]. In preterm infants, provision of high amounts of omega-3 LC-PUFA without a concomitant supply of ARA has been associated with adverse effects on growth [62, 63]. Further concerns regarding the effects of a high supply of DHA without increasing ARA intakes on infants are raised by the findings of a randomized controlled trial assigning term infants to formula providing either no LC-PUFA or different levels of 0.32, 0.64, and 0.96% DHA at the same ARA level of 0.64% [64]. The investigators performed developmental testing of the participating children up to the age of 6 years. Positive effects in tests on word production, a card sorting task, and an intelligence test were observed with the lower DHA dose. However, performance of children assigned to the highest DHA dose of 0.96% but with a reduced ratio of dietary ARA to DHA was attenuated in the MBCDI Word Production Test and the Dimensional Change Card Sort Test at the highest DHA level, and it was attenuated at the two highest DHA levels in the Peabody Picture Vocabulary Test [64]. Thus, in contrast to what might have been expected, an increase of formula DHA contents above 0.32% did not further improve or at least stabilize developmental outcomes, but actually had adverse effects which might well be due to the reduced dietary ARA to DHA ratios provided with the higher DHA levels.

The effects of equivalent formulae with similar DHA and ARA contents on brain composition were tested in infant baboons. Brain composition in various regions was analyzed. The formula with about 1% DHA induced a trend to lower ARA levels in the retina and all the 8 regions of the brain analyzed, with significantly reduced ARA values in the globus pallidus and the superior colliculus, even though the formula contained 0.64% ARA. These observations raise serious concerns that infant formula with high contents of DHA but lack of ARA may induce adverse effects on brain composition and related functional outcomes.

These findings in human infants and in nonhuman primates question the suitability and safety of the compositional requirements stipulated by the new European legislation, i.e. to provide infant formula from birth with up to 1% of fatty acids as DHA without a proportional increase in the intake of ARA. It is generally agreed upon that any major change in infant formula composition should be subjected to a full preclinical and clinical evaluation of nutritional adequacy and safety prior to the wide use and market introduction of such a modified formula [65–70]. Therefore, it appears to be inappropriate and premature to market formula for term infants from birth with 20–50 mg/100 kcal DHA without added ARA in the absence of accountable data on the suitability and safety from a thorough clinical evaluation of this novel approach [50].

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**Conclusion**

In addition to meeting the infant needs for energy and essential vitamins and PUFA, human milk lipids provide a mixture of MFGM, complex lipids, and bioactive compounds that may have important biological roles in the breastfed infant, for example with regard to the development of nervous and immune functions. Further studies defining the specific components responsible for such effects and the underlying mechanisms could help to design the best options of nutritional interventions. Methodological progress in the field of metabolomics and lipidomics using liquid chromatography coupled with triple mass spectrometry now allows to determine detailed profiles of molecular species of complex lipids in milk as well as in extremely small sample volumes of infant serum or plasma (e.g. 10 μL) with high quantitative precision [71–74]. Such lipidomic measurements can serve to provide markers for tissue composition [75] and were shown to be associated with important clinical endpoints in children and adults [76–78]. It is therefore likely that the use of these sophisticated and detailed analytical methods, if combined with appropriate bioinformatics strategies, provide the opportunity to obtain better insights into the physiological roles of complex lipids in early life, which may lead to further improvements in nutritional strategies. Progress in biotechnology and food technology offers new avenues for preparing lipid components that can more closely mimic the complex lipid body provided with breastfeeding. Careful exploration and evaluation of the

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Human Milk Lipids

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short- and long-term impact in infants could potentially lead to implementation of major improvements for the feeding of infants who cannot be breastfed. Opportunity also exists in improving our understanding of the optimal supply of LC-PUFA in early and later infancy and in the underlying mechanisms and mediators of their effects, e.g. on neurodevelopment and behavior, immune-related health outcomes, such as allergy and asthma, and pulmonary function.

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Koletzko

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Human Milk Lipids


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The immune benefit of breastfeeding has been attributed in part to the diverse bioactive components in human milk

Human Milk Oligosaccharides Influence Neonatal Mucosal and Systemic Immunity
by Sharon M. Donovan and Sarah S. Comstock

**Key insights**

Human milk confers multiple layers of protection to the newborn by providing bioactive components that protect the infant from pathogenic infection, facilitate intestinal and immune development, and support healthy gut microbes. An important bioactive component of human milk are the human milk oligosaccharides (HMOs). HMOs are a complex mixture of indigestible carbohydrates with a high degree of structural diversity and represent one of the largest groups of bioactive components in human milk.

**Current knowledge**

HMOs are a family of soluble glycans that are sialylated or fucosylated and provide carbon sources for gut bacterial species that colonize breastfed infants. Through their actions on the gut, HMOs directly and indirectly affect the infant’s mucosal and systemic immunity. A large number of studies have shown that HMOs can influence the proliferation and maturation of intestinal cells (such as crypt cells and goblet cells). Furthermore, HMOs can also modulate gene expression in the intestinal epithelium. Altogether, these affect the function of the intestinal barrier, which in turn regulates local and systemic immunity.

**Practical implications**

Human milk contains a higher concentration and a greater structural diversity and degree of fucosylation compared to the milk oligosaccharides in other species, including cow’s milk from which many infant formulae are derived. Commercially produced HMOs are becoming increasingly available, and evidence suggests that supplementing infant formulae with HMOs is safe and beneficial for human infants. There are also potential applications of HMOs as prophylactic and therapeutic treatments for those who are immune compromised and at high risk of infection.

**Recommended reading**

Human Milk Oligosaccharides Influence Neonatal Mucosal and Systemic Immunity

Sharon M. Donovana  Sarah S. Comstockb

a Department of Food Science and Human Nutrition, University of Illinois, Urbana, IL, and b Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI, USA

Key Messages
- Human milk oligosaccharides (HMO) are a predominant component of human milk and are comprised of diverse structures that are neutral or acidic and some forms are sialylated or fucosylated, which contributes to their biological functions.
- HMO protect the infant from pathogenic infections, facilitate the establishment of the gut microbiota, promote intestinal development, and stimulate immune maturation.
- Some types of HMO are now commercially available and are being added to infant formula alone or in combination with other prebiotics.

Keywords
Human milk oligosaccharides · Immunity · Infant

Abstract
The immune system of the infant is functionally immature and naive. Human milk contains bioactive proteins, lipids, and carbohydrates that protect the newborn and stimulate innate and adaptive immune development. This review will focus on the role human milk oligosaccharides (HMO) play in neonatal gastrointestinal and systemic immune development and function. For the past decade, intense research has been directed at defining the complexity of oligosaccharides in the milk of many species and is beginning to delineate their diverse functions. These studies have shown that human milk contains a higher concentration as well as a greater structural diversity and degree of fucosylation than the milk oligosaccharides in other species, particularly bovine milk from which many infant formulae are produced. The commercial availability of large quantities of certain HMO has furthered our understanding of the functions of specific HMO, which include protecting the infant from pathogenic infections, facilitating the establishment of the gut microbiota, promoting intestinal development, and stimulating immune maturation. Many of these actions are exerted through carbohydrate-carbohydrate interactions with pathogens or host cells. Two HMOs, 2′-fucosyllactose (2′FL) and lacto-N-neotetraose (LNnT), have recently been added to infant formula. Although this is a first step in narrowing the compositional gap between human milk and infant formula, it is unclear whether 1 or 2 HMO will recapitulate the complexity of actions exerted by the complex mixture of HMO ingested by breastfed infants. Thus, as more HMO become commercially available, either isolated from bovine milk or chemically or microbially synthesized, it is anticipated that more oligosaccharides will be added to infant formula either alone or in combination with other prebiotics.

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Background

The human infant enters the world with a functionally naïve immune system affecting both adaptive and innate immune responses [1], which leaves the newborn at high risk for common infections. Postnatal immune maturation is stimulated by antigenic exposures and host-microbe interactions [1, 2]. How and what the infant is fed influences the development and competence of the immune system [3–5]. Human milk protects the infant during this vulnerable period by providing bioactive components that protect the infant from pathogenic infection, support intestinal development, promote barrier function, stimulate immune development, facilitate immune tolerance, and feed gut microbes [2–5]. Thus, human milk supplies multiple layers of protection for the infant (Fig. 1).

Breastfeeding, particularly exclusive breastfeeding for 6 months or more, relative to formula-feeding, decreases the incidence and/or severity of infectious diseases [6]. Many diseases with infectious and immune components in their etiology, including diarrhea, respiratory and urinary tract infections, otitis media, bacteremia, and necrotizing enterocolitis occur less often in breast- than formula-fed infants [6, 7]. Breastfeeding has also been implicated in reducing the incidence of other diseases involving the immune system and immune tolerance, such as inflammatory bowel disease, celiac disease, asthma, allergy, type 1 diabetes, as well as acute lymphoblastic and acute myeloblastic leukemias [6, 8]. These benefits may be mediated in part through effects of breastfeeding on the intestinal microbiota [8, 9], which in turn stimulates maturation and specificity of the neonatal mucosal and systemic immune systems [2].

The immune benefit of breastfeeding has been attributed in part to the diverse bioactive components in human milk [2–5]. A strong case can be made for a key role of human milk oligosaccharides (HMO) in neonatal immune defense and maturation. As will be described below, HMO are present in high concentrations in human milk, exist in an incredible structural diversity [10–13], and confer host protection and mediate immune responses through a number of mechanisms [14, 15].

HMO Content and Composition

HMO are complex soluble glycans that are predominantly present in free form in milk. These glycans are synthesized from 5 basic monosaccharides: galactose, glucose, N-acetylglucosamine, fucose, and the sialic acid derivative N-acetyleneuraminic acid [10, 11]. With few exceptions, all HMO carry lactose (Galβ1–4Glc) at the reducing end, which can be elongated in β1–3 or β1–6 linkage by 2 different disaccharides, either Galβ1–3GlcNAc (type 1 chain) or Galβ1–4GlcNAc (type 2 chain) [11].

The HMO content has been reported in the range of 1–10 g/L in mature milk and 15–23 g/L in colostrum [10–13]. In term breast milk, ~35–50% of HMO are fucosylated, 12–14% are sialylated, and 42–55% are nonfucosylated neutral HMO [10–13] (Table 1). However, HMO composition is influenced by maternal genetics, including secretor and Lewis Blood Group status [10, 11]. HMO fucosylation is mediated by the 2 fucosyltransferases FUT2 (secretor gene) and FUT3 (Lewis gene). Nonsecretor mothers, who lack a functional FUT2 enzyme and represent about 30% of women worldwide, produce milk lacking in α1-2-fucosylated oligosaccharides like 2′-fu-
cosyllactose (2′FL) and lacto-N-fucopentaose (LNFP) I [10, 11]. The absence of these compounds may have functional consequences. For example, infants consuming milk produced by women who are nonsecretors exhibit delayed colonization of bifidobacteria, higher abundance of Streptococcus taxa, and have functional differences in the metabolic activity of their microbiota [16]. Infants fed milk from nonsecretor mothers are at higher risk for diarrheal diseases [17].

**HMO and the Microbiome**

The development of the infant gut microbiota is a sequential process that begins in utero and continues during the first 2–3 years of life. Microbial composition and diversity is shaped by host genetics and multiple environmental factors, of which diet is a principal contributor [8, 9]. Studies conducted over the past decade have shown that specific *Bacteroides* and *Bifidobacterium* species that commonly colonize breastfed infants efficiently utilize HMO as carbon sources. This is particularly true of *B. longum* ssp. *infantis* (B. *infantis*), which is a predominant gut microbe in most breastfed infants [18]. The discovery of a genomic island in *B. infantis* that encodes specific enzymes for the metabolism of HMO supports an adaptation of this species to the intestinal milieu of the breastfed infant [18, 19]. Indeed, a recent study in human infants fed formula supplemented with 2′FL (1 g/L) and LNNnT (0.5 g/L) demonstrated that the global microbiota composition of infants fed formulae with 2′FL and LNNnT was significantly different to that of infants fed nonsupplemented formula (p < 0.001) at the genus level and closer to that of breastfed infants at 3 months of age [20]. In addition, *Bifidobacterium* was more abundant (p < 0.01), whereas *Escherichia* and unclassified Peptostreptococcaceae were less abundant in infants fed formula with 2′FL and LNNnT compared to infants fed nonsupplemented formula, and these levels were closer to those observed in breastfed infants [20]. Furthermore, the concentrations of several important metabolites in stool (propionate, butyrate, and lactate) in infants fed the HMO-supplemented formula were more similar to those of breastfed infants [20].

Previously, we have shown that HMO fermentation by neonatal pig microbiota produced short-chain fatty acids and promoted the growth of beneficial bacteria in vitro [21] and in vivo [22]. Gut bacteria and the immune response, particularly the gastrointestinal immune response, are tightly interrelated [23]. Thus, in this animal model, HMO-induced changes in the gut bacterial populations of the pigs could alter the course of an intestinal infection [24] which in turn would alter the immune response [22]. Alternatively, the change in the gut bacteria could directly affect the immune system of these animals [2]. Additional ways whereby HMO may mediate neonatal immunity are summarized in the following section.

**HMO as Immune Modulators**

Summarized in Figure 2 are the results of an accumulating body of evidence showing that HMO indirectly and directly influence infant mucosal and systemic immune function. In general, intestinal health and barrier function are considered a first line of defense in innate immunity. Cell proliferation takes place in the crypts, and cells differentiate as they migrate up the villus-crypt axis, with the exception of Paneth cells, which migrate down to the base of the crypt. HMO reduce intestinal crypt cell proliferation [25, 26], increase intestinal cell maturation [26], and increase barrier function [26] (indicated by 1–3 in

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**Table 1. Concentrations of major HMO in human milk [10 – 13]**

<table>
<thead>
<tr>
<th>Categories of HMO (% total)</th>
<th>Oligosaccharide</th>
<th>Mean concentration (range), g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucosylated (35–50%)</td>
<td>2′FL</td>
<td>2.7 (1.88 – 4.9)</td>
</tr>
<tr>
<td></td>
<td>3′FL</td>
<td>0.5 (0.25 – 0.86)</td>
</tr>
<tr>
<td></td>
<td>LNFP I</td>
<td>0.122 (0.106 – 0.145)</td>
</tr>
<tr>
<td></td>
<td>LNFP II + III</td>
<td>0.156 (0.120 – 0.161)</td>
</tr>
<tr>
<td>Sialylated (12–14%)</td>
<td>3′SL</td>
<td>0.2 (0.1 – 0.3)</td>
</tr>
<tr>
<td></td>
<td>6′SL</td>
<td>0.5 (0.2 – 1.22)</td>
</tr>
<tr>
<td>Nonfucosylated neutral (42–55%)</td>
<td>LNNnT</td>
<td>0.3 (0.17 – 0.45)</td>
</tr>
</tbody>
</table>

2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose; HMO, human milk oligosaccharides; LNFP, lacto-N-fucopentaose; LNNnT, lacto-N-neotetraose.
Fig. 2. Potential mechanisms whereby human milk oligosaccharides (HMO) influence host immune function. HMO affect innate immunity through the epithelial barrier: HMO reduce intestinal crypt cell proliferation (1), increase intestinal cell maturation (2), increase barrier function (3), and may influence goblet cell function (4), as has been shown for galacto-oligosaccharides. In addition, HMO affect epithelial immune gene expression both directly (5) and indirectly through the microbiota (6). HMO serve as prebiotics to promote the growth of healthy bacteria, including *Bifidobacteria* and *Bacteroides* species (7), and HMO inhibit infections by bacteria and viruses by either binding to the pathogen in the lumen or by inhibiting binding to cell-surface glycan receptors (8). HMO affect immune cell populations and cytokine secretion (9). HMO are also absorbed into the blood (10), where they affect binding of monocytes, lymphocytes, and neutrophils to endothelial cells (11) and formation of platelet-neutrophil complexes (12).
A layer formed by mucus glycoproteins or mucins produced by goblet cells acts as a lubricant and a protective physical barrier between the intestinal epithelium and the luminal contents (indicated by 4 in Fig. 2). HMO may influence goblet cell function, as has been shown for galacto-oligosaccharides (GOS) [27]. HMO affects epithelial immune gene expression both directly [28–30] and indirectly through the microbiota [31] (indicated by 5 and 6 in Fig. 2, respectively). As noted above, HMO serve as prebiotics to promote the growth of healthy bacteria, including Bifidobacteria and Bacteroides genera [32] (indicated by 7 in Fig. 2), and HMO inhibit infections by bacteria and viruses by either binding to the pathogen in the lumen or by inhibiting binding to cell-surface glycan receptors [14–15, 22] (indicated by 8 in Fig. 2). Additionally, dietary oligosaccharides decorate the intestinal lining contributing to the intestinal glycan repertoire [33]. HMO also contribute to epithelial barrier function by supporting the growth of B. infantis in the infant gut [10, 18]. B. infantis produces peptides that have been shown to normalize intestinal permeability through enhanced tight junction protein expression in a mouse model of colitis [34]. It is likely that HMO support other bacterial species that are important for the maintenance of gut integrity. These changes in intestinal barrier function would, in turn, alter both the local and systemic immune system [35]. HMO affect immune cell populations and cytokine secretion [22, 36] (indicated by 9 in Fig. 2). Some HMO are also absorbed into the blood stream [37–39] (indicated by 10 in Fig. 2), where they exert systemic effects by binding of monocytes, lymphocytes, and neutrophils to endothelial cells [40] (indicated by 11 in Fig. 2) and formation of platelet-neutrophil complexes [41] (indicated by 12 in Fig. 2). Readers are referred to a recent review by Kulinich and Liu [15] for additional discussion of this topic.

**Carbohydrate Binding as a Potential Mechanism of HMO in the Immune System**

Carbohydrates and carbohydrate-binding proteins play an important role in immune responses. Cells have unique glycan signatures made from combinations of specific glycan motifs that are engaged when a cell contacts another cell or other components of its environment [42, 43]. However, many of the glycan motifs found on mammalian cells are also found on microbes and in food, including human milk. These similarities provide opportunities for host-microbe-HMO interactions.

Lectins are carbohydrate-binding proteins on the surfaces of mammalian cells that translate recognition of specific motifs and the spatial presentation of those motifs into action. Lectins are grouped according to their carbohydrate recognition domains (CRD) [42, 43]. There are at least a dozen CRD identified in mammals, but 3 classes of lectins related to the influence of HMO on immune responses are C-type lectins, siglecs (sialic acid-binding Ig-like lectins), and galectins.

C-type lectins require calcium to function and include selectins, mannose-binding lectin, and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). C-type lectin receptors on the surface of dendritic cells (DC) determine whether the cell will induce tolerance rather than lymphocyte activation [44]. DC-SIGN is of particular interest with regard to mechanisms by which HMO can influence immunity because it has a CRD specific for fucose units. Furthermore, DC-SIGN is expressed by cells in the gastrointestinal tracts of infants [45]. These intestinal cells are likely antigen-presenting cells as DC-SIGN is expressed by antigen-presenting cells, specifically DC [43]. Although interactions between fucosylated ligands and DC-SIGN contribute to immune tolerance, the cellular response ultimately depends upon the other ligand-receptor reactions occurring simultaneously [43].

Siglecs are sialic acid-binding lectins most commonly found on subsets of immune cells [46]. There are at least 16 siglec expressed by different leucocyte populations, which include sialoadhesin (siglec-1), CD22 (siglec-2), myelin-associated glycoprotein (MAG, siglec-4), siglec-15, and CD33-related siglecs. Siglec specificity derives from differences in secondary binding sites [43]. Siglecs are endocytic cell surface receptors that carry cargo between the cell surface and intracellular vesicles; these receptors are mainly expressed on cells involved in antigen processing and presentation [43]. Furthermore, sialic acid-containing molecules can gain entry to macrophages by binding to siglecs on the cell surface [46]. On mammalian cells, some sialic acid-containing glycans function as self-associated molecular patterns and prevent immune responses to nonpathogenic stimuli. Ligation of particular siglecs stimulates the production of the immunoregulatory cytokine interleukin (IL)-10 [47].

Galectins are important for cell turnover and immune regulation. The CRD of galectins is specific for β-galactosides. When cells are desialylated, the density of exposed galactose moieties on the cell surface increases. For example, naïve T cells express CD45 with an α-2,6-linked sialic acid. The amount of α-2,6-linked sialic acid is re-
HMO as Modulators of Mucosal Immunity

Intestinal cell lines have been used to determine effects of HMO on immune-related gene expression and protein production. These cells have been co-incubated with oligosaccharides [28], bacteria [48], or lipopolysaccharides (LPS) to model a bacterial infection [29]. Co-incubation of Bifidobacterium with cells of the Caco-2 intestinal cell line and HMO resulted in downregulation of intestinal cell genes related to chemokine activity compared to co-incubation with glucose or lactose [29]. Conversely, in the absence of a bacterial co-stimulant, HMO increased expression of several chemokines by the HT-29 cell line [28]. Additional work in T84 and HCT8 intestinal cell lines showed that complex mixtures of HMO as well as 2′FL reduced signatures of intestinal inflammation [29].

Table 2. Immune-related outcomes of HMO feeding studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Study design</th>
<th>Major findings</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Human infants</td>
<td>Healthy singleton infants enrolled by 5 days of life and fed formulae to 4 months of age</td>
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<tr>
<td></td>
<td>- Breastfed</td>
<td>Breastfed infants and infants fed either formula with 2′FL were similar and had lower plasma inflammatory cytokines than infants fed the control formula</td>
<td>53</td>
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<td></td>
<td>- Formula + 2.4 g/L GOS</td>
<td>In ex vivo RSV-stimulated PBMC cultures, cells from breastfed infants were not different from those of either of the groups fed formula with 2′FL, but secreted less TNF-α and IFN-γ and tended to have lower IL-1Ra, IL-6 and IL-1β than cells from infants fed the control formula</td>
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<td>- Formula + 2.2 g/L GOS + 0.2 g/L 2′FL</td>
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<td>- Formula + 1.4 g/L GOS + 1.0 g/L 2′FL</td>
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<td></td>
<td>PBMC isolated at 6 weeks of age</td>
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<td>Human infants</td>
<td>Healthy singleton infants enrolled by 14 days of life and fed experimental formulae to 6 months of age, and standard follow-up formula to 12 months</td>
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<tr>
<td></td>
<td>- Formula</td>
<td>Infants fed HMO-supplemented formula had significantly fewer parental reports of:</td>
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<td></td>
<td>- Formula + 1.0 g/L 2′FL + 0.5 g/L LNnT</td>
<td>- Bronchitis through 4, 6, and 12 months</td>
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<td>Colostrum-deprived</td>
<td>15-day feeding study</td>
<td>- Lower respiratory tract infection through 12 months</td>
<td>22</td>
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<tr>
<td>piglets</td>
<td>- Formula</td>
<td>- Antibiotics use through 6 and 12 months</td>
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<td></td>
<td>- Formula + 4 g/L HMO (40% 2′FL; 35% LNnT; 10% 6′SL; 5% 3′SL; 10% free SA)</td>
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<tr>
<td>Adult female</td>
<td>E. coli infection model</td>
<td>HMO resulted in shorter duration of diarrhea and higher ileal IFN-γ and IL-10 mRNA expression than formula, but similar concentrations of RV-specific IgG and IgM as formula</td>
<td>29</td>
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<tr>
<td>C57BL/6 mice</td>
<td>- 0.25% DSS orally for days 0–3</td>
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<td>- 2′FL (100 mg or vehicle by oral gavage days 0–4)</td>
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<td>- 20 mg streptomycin by oral gavage on day 4</td>
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<td>- Infected with OSU strain RV on day 10 and analyzed on day 15</td>
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<tr>
<td>Adult male</td>
<td>Food allergy treatment model</td>
<td>2′FL prevented body weight loss and reduced AIEC colonization, colonic inflammation, crypt cell CD14 expression, and IL-6, IL-17, and TNF-α production in response to AIEC infection</td>
<td>51</td>
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<tr>
<td>Balb/c mice</td>
<td>- IP sensitized to OVA</td>
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<td>- 2 weeks later (day 27), oral gavage daily (1 mg in 200 μl PBS)</td>
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<td></td>
<td>- 2′FL</td>
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<td></td>
<td>- 6′SL</td>
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<td></td>
<td>- Lactose</td>
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<td></td>
<td>- Day 28 oral challenge with OVA (50 mg) every 3 days until day 43</td>
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<td></td>
<td>2′FL and 6′SL attenuated diarrhea and hypothermia induced by OVA challenge, reduced intestinal mast cell numbers and passive cutaneous anaphylaxis, and increased Peyer’s patch T regulatory cells and CD11c+CD103+ DC 6′SL increased OVA-specific IgG2a and MLN T regulatory cells Splenocytes from 6′SL-treated mice produced more IFN-γ and IL-10 but less TNF Splenocytes from 2′FL-treated mice produced less IFN-γ</td>
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2′FL, 2′-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialylactose; AIEC, adherent-invasive E. coli; DC, dendritic cells; DSS, dextran sodium sulfate; GOS, galacto-oligosaccharides; HMO, human milk oligosaccharides; IFN, interferon; IP, intraperitoneal infection; Ig, immunoglobulin; IL, interleukin; LNnT, lacto-N-neotetraose; MLN, mesenteric lymph nodes; OSU, Ohio State University; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RSV, respiratory syncytial virus; RV, rotavirus; SA, sialic acid; TNF, tumor necrosis factor.
HMO have been demonstrated to affect the course of a gastrointestinal viral infection. In an acute rotavirus (RV) infection model where a 21-day-old piglet’s ileum was isolated in situ, intestinal loops co-treated with HMO and RV had reduced non-structural protein-4 (NSP-4) mRNA expression indicating that HMO can reduce RV replication [49]. Intestinal cytokine and chemokine expression, however, was not affected. Both neutral and acidic HMO decreased NSP4 intestinal mRNA expression in the in situ model, whereas only acidic HMO effectively inhibited RV infectivity in an in vitro model [49].

**HMO as Modulators of Systemic Immunity and Protection from Infection**

HMO are detected in the plasma of infants fed human milk at concentrations of 1–133 mg/L [37, 39], suggesting the potential for dietary HMO to directly affect immune cells circulating in the blood. As discussed above, many immune receptors recognize the oligosaccharide structures of their glycoprotein ligands [14, 15]. Because a subset of HMO is structurally similar to selectin ligands [14], it is likely that HMO can bind directly to immune cells and trigger signaling that results in changes to immune cell populations and functions. For instance, the P- and E-selectins recognize sialyl-Lewis x (sLeX), a glycan moiety of several HMO [11]. Additionally, fucosylation and sialylation, 2 enzymatic modifications common to HMO, enable binding to selectins [50]. HMO-induced disruption of immune protein-carbohydrate interactions reduced neutrophil rolling [40] and activation [41]. HMO directly affect immune cell proliferation and cytokine production in ex vivo experiments with peripheral blood mononuclear cells (PBMC) from neonatal pigs [36]. Stimulation with isolated HMO stimulated production of the regulatory cytokine IL-10 [36]. Others observed that the acidic HMO induce IL-10 production; additionally, they found that acidic HMO induce IFN-γ from ex vivo stimulated human cord blood mononuclear cells [45]. Isolated HMO enhanced proliferation of PBMC stimulated with a T-cell mitogen, phytohemagglutinin (PHA), and sialylated HMO enhanced proliferation of PBMC stimulated with the B-cell mitogen LPS [36]. In contrast, 2′FL inhibited proliferation of unstimulated PBMC cultured for 3 days. Thus, the response to HMO may depend on the state of the infant. In the unstimulated state, HMO dampen proliferation, whereas HMO enhance proliferation in response to a mitogenic stimuli.

To date, very few studies have fed HMO and analyzed immune outcomes [22, 29, 30, 51–54] (Table 2). Piglets [55] have been fed 2′FL, but only growth and toxicological outcomes were reported. A recently published paper described immune outcomes in human infants fed formula containing 2.4 g/L GOS, 2.2 g/L GOS + 0.2 g/L 2′FL, or 1.4 g/L GOS + 1.0 g/L 2′FL compared to a breastfed reference control [53]. Infants were fed the formula from 5 days to 4 months of age, and blood samples were obtained at 6 weeks of age for cytokine analysis, immune cell phenotyping, and ex vivo stimulation of isolated PBMC. Breastfed infants and infants fed either formula with 2′FL were similar and had lower plasma inflammatory cytokines than infants fed the control formula. In addition, cytokine secretion by PBMC from breastfed infants and infants fed either 2′FL-containing formula that were stimulated ex vivo with respiratory syncytial virus was similar and secreted less tumor necrosis factor-α and interferon-γ and tended to have lower IL-1Ra, IL-6, and IL-1β than cells from infants fed the control formula [53].

Another recent study in human infants evaluated the effect of formula supplemented with both 2′FL (1.0 g/L) and LNnT (0.5 g/L) compared to an unsupplemented formula. Infants were fed the formulae from 14 days to 6 months of age, after which they were switched to a standard follow-on formula and followed until 12 months of age. Infants fed the HMO-supplemented formula had significantly fewer parental reports (p = 0.004–0.047) of: bronchitis through 4 months (2.3 vs. 12.6%), 6 months (6.8 vs. 21.8%), and 12 months (10.2 vs. 27.6%); lower respiratory tract infection (AE cluster) through 12 months (19.3 vs. 34.5%); antipyretics use through 4 months (15.9 vs. 29.9%); and antibiotics use through 6 months (34.1 vs. 49.4%) and 12 months (42.0 vs. 60.9%) than those fed the standard formula [54].

Several studies in animal models support the reduced incidence of infection in human infants fed formula with HMO. In mice infected with *Escherichia coli*, once daily oral gavage with 100 mg, 2′FL prevented body weight loss and reduced colonization with adherent-invasive *E. coli*, colonic inflammation, crypt cell CD14 expression, as well as IL-6, IL-17, and tumor necrosis factor-α production in response to adherent-invasive *E. coli* infection compared to mice treated with vehicle [29]. Mice fed 2′FL and subjected to ileocecal resection gained more weight and had greater crypt depth and villus height at the site of transection than nonsupplemented mice [30]. The 2′FL-fed mice also had upregulated mucosal immune response genes in the distal small bowel [30]. The studies where pigs and human infants were fed the HMO have focused on 2′FL, which is readily available in large quantities at reasonable cost, and fucosylated oligosaccharides have been shown...
to feed specific beneficial classes of bacteria during intestinal inflammatory events [56]. Given what is known about the effects of other HMO, these compounds also should be used in feeding studies when available in sufficient quantities.

Only 1 in vivo study used a complex mixture of HMO and assessed immune outcomes. In that report, neonatal pigs fed a diet containing 4 g/L HMO, consisting of 40% 2′FL, 10% 6′-sialyllactose (6′SL), 35% lacto-N-neotetraose (LNnT), 5% 3′-sialyllactose (3′SL), and 10% free sialic acid, had a reduced duration of diarrhea, in response to RV infection to 48.8 ± 9.8 h versus 80.6 ± 4.5 h in pigs fed nonsupplemented formula [22]. Ileal tissue from the pigs fed HMO contained greater IFN-γ (produced by Th1 cells) and IL-10 (an anti-inflammatory cytokine) mRNA than that from pigs fed formula [22].

In a mouse model of food allergy, 2′FL and 6′SL administered via oral gavage reduced symptoms in mice sensitized to ovalbumin, an egg protein [51]. Specifically, ovalbumin-stimulated splenocytes from mice treated with 6′SL produced more IL-10 and less IFN-γ than those from untreated mice. Furthermore, 2′FL- or 6′SL-treated mice had more regulatory immune cells in their intestinal immune tissues than untreated mice. Interestingly, neither 2′FL nor 6′SL affected intestinal T regulatory cells when administered to nonsensitized mice [51]. This exemplifies the necessity of identifying an appropriate challenge model to assess the effects of dietary compounds on the immune system. In mice, the milk oligosaccharides LNFP III and LNnT are Th2-biasing and suppress Th1 responses [57]. Recently, it has been reported that human infants who were fed human milk with low LNFP III concentrations (<60 μM) were 6.7-times (95% CI 2.0–22) more likely to become affected with cow’s milk allergy when compared to infants receiving milk with high LNFP III concentrations [58].

Another approach using knockout mice showed that SL-containing compounds can directly affect gastrointestinal mucosal immunity [52, 59]. In one study, the presence of 3′SL in the milk increased the number of immune cells infiltrating the gut in IL-10 null mice [52]. Furthermore, supplementation with 3′SL increased colitis severity in newborn IL-10 and St3gal4 (the enzyme that synthesizes 3′SL) null mice, and cross-fostering wildtype mice to deficient dams reduced colitis severity. One caveat of this work is that it was conducted in the absence of endogenous IL-10 production, whereas other in vivo studies have demonstrated that some HMO increase intestinal IL-10 [22, 51]. 3′SL is a product of several pathogenic bacteria [60] and the conformation (α2,3-link between sialic acid and galactose) on pathogenic bacteria and in human milk is the same. 3′SL is recognized by DC and generates an immune response through the TLR4 signaling pathway [61]. These results suggest that the presence of 3′SL increases the inflammatory response through direct effects on DC. When TLR4 was absent, 3′SL was less effective at inducing DC activation. However, those DC also demonstrated a minimal increase in CD40 expression suggesting that at least one other 3′SL-sensing mechanism, albeit much less efficient than the TLR4 pathway, exists on DC. TLR4 is the receptor for E. coli LPS. Another link between 3′SL and TLR4 is explained in a newer paper, where it is demonstrated that 3′SL stimulates the proliferation of the intestinal E. coli population and that this overgrowth of E. coli is responsible for exacerbation of dextran sulfate sodium colitis through release of proinflammatory cytokines from intestinal DC [62]. These examples demonstrate the complexity of the relationships between oligosaccharides, the gut bacteria and the immune system.

**Conclusion**

The rich diversity of HMO has the potential to modulate both innate and adaptive neonatal immunity. Findings from in vitro experiments and animal models show that HMO directly interact with gastrointestinal epithelial cells as well as with mucosal and systemic immune cells to modulate immune function. HMO also beneficially shape the microbiome of the breastfed infant. The increased availability of HMO from commercial sources as well as accumulating evidence demonstrating that formula supplemented with HMO is safe and may confer benefits for human infants have led to the recent addition of 2′FL alone or in combination with LNnT to infant formulae. In addition, due to their beneficial effects on immune function and host defense, HMO may also be beneficial for other segments of the population who are immune compromised or at high risk for infection. There are limited studies in which animals or humans have been fed HMO. Additionally, few studies have assessed the effects of feeding complex mixtures of HMO on the immune response. Thus, future research is needed to delineate mechanisms and to fully realize the potential for HMO to benefit infant immune function.

**Disclosure Statement**

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