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Human milk as a complex natural product

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Breastfeeding is one of the most effective ways to promote child health. However, characterizing the chemistry that fortifies the benefits of breastfeeding remains a grand challenge. Current efforts in the community are focused on characterizing the roles of the different carbohydrates, proteins, and fats in milk. The goal of this review is to highlight and describe current knowledge about the major classes of macromolecules in human milk and their potential role in infant health and wellness.

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1 Introduction

Traditionally, natural products have played a central role in the intellectual and experimental growth of organic chemistry. Undeniably, a disproportionate amount of clinical therapeutics are natural products or are natural product derived. On occasion, a natural product can possess the requisite pharmacological characteristics to render it a clinically beneficial medication. More often, however, are examples where a natural product serves as "the lead", providing a structural platform, which the organic chemist can build upon to derive new chemical matter and a valuable therapeutic.

Historically, the most important sources for natural products have been plants, aquatic invertebrates, and soil-bound microorganisms. While these studies have produced a bounty, it is likely that many valuable natural compounds have escaped discovery. Moreover, we have limited what we define as a natural product. It is in this vein that we draw the reader's attention to human milk. At the molecular level, human milk is a complex mixture where the composition reflects the secretory activity of the mother's mammary gland. Blood group also plays a major role in milk composition, particularly in the context of oligosaccharide profile, *vida infra*. Previously, human milk has been considered as a source of new therapeutics. In this writing, we make the case for human milk, in its homogeneous form, *as* a natural product.

During early life, milk fulfills all nutritional requirements for the developing infant. Given its dynamic nature and ability to meet the needs of the child in real time, the World Health Organization and United Nations Children's Fund recommend exclusive breastfeeding for at least 6 months after birth and to continue for up to 2 years of age or longer.[1] Recently, infant food products have been developed that share greater homology to human milk, with many being supplemented with small amounts of naturally occurring compounds such as human milk oligosaccharides (HMOs).[2] While these products feature molecules native to human milk, a homologous, synthetic alternative to human milk does not exist. Consequently, it is imperative to critically evaluate the natural products that make human milk the gold standard for infant nutrition.

2 The origin of human milk

In 1758, Linnaeus named animals with the ability to produce milk *Mammalia*. The evolution of the mammary gland, the

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glandular organ that generates milk, is difficult to establish due to a lack of evidence supporting the origin of soft tissue organs from fossils. However, it is well established that lactation appears to be a reproductive feature that predates the evolution of mammals. A persuasive theory for the evolution of the mammary gland and lactation was elegantly curated by Oftedal.[3] The molecular components of human milk arise from a variety of sources – some are produced and secreted by the epithelium of the mammary gland. Others are produced by cells found in the milk. Lastly, select molecules are taken from maternal blood and transported across the mammary epithelium into the milk.

3 Stages of lactation

Colostrum is the initial type of milk produced by a mother. While this "first milk" can vary in color, it is often deep yellow or orange due to its high concentration of β-carotene. Colostrum, produced in low volume during the immediate postnatal period, is rich in immune-enhancing and developmental components such as immunoglobulins (*e.g.* secretory IgA), glycoproteins (*e.g.* lactoferrin), leukocytes, and epidermal growth factor.[4–6] Interestingly, colostrum has a lower concentration of fat and carbohydrate, compared to mature milk, which hints to its primary role as being protective and developmental rather than nutritional. As tight junction closure commences in the epithelium of the mammary gland, the concentration of lactose increases (triggered by a decline in the sodium to potassium ratio) and the production of transitional milk begins. The timing of this secretory activation process varies among women, but typically occurs over the first week postpartum. While intermediate milk has molecular homology with colostrum, it also represents a period of increased milk production to support the nutritional and developmental needs of the infant. After 14 days postpartum, milk is considered mature. Contrasting to the dynamic nature of milk observed early in life, the molecular components remain relatively stable once milk reaches maturity.

4 Human milk composition

The composition of human breast milk is well-established (Table 1). Milk is *ca.* 87% water. The major macromolecules can be organized into three categories: carbohydrates (7%), fat (4%), and protein (1%). Counter to infant formula, the nutritional and protective molecules in breast milk are dynamic and vary depending on maternal diet and health, mammary gland physiology, and the needs of the child.

Table 1 Approximate composition of mature human breast milk

Component	Concentration (g L^{-1})	% (w/v) of breast milk
Water	—	87%
Carbohydrates	70	7%
Fat	35–40	4%
Protein	11	1%
Growth factors, hormones, cytokines, *etc.*	0.9	<1%

4.1 Carbohydrates

4.1.1 Lactose. Lactose is the 3rd most abundant molecule found in human breast milk, with an average concentration of 70 g L^{-1}, notably higher than the 50 g L^{-1} found in cow's milk.[7] Biosynthetically, lactose is produced in the golgi of mammary glands cells through the linkage of UDP-galactose and glucose (Fig. 1A). This process begins with the transport of extracellular glucose into the cytosol where it is either retained as glucose or activated to UDP-glucose. UDP-glucose is subsequently converted to UDP-galactose. Both UDP-galactose and glucose are then transported into the golgi where the lactose synthase complex facilitates their conjugation.

The lactose synthase complex consists of two components: the "A" protein and the "B" protein. The "A" protein is a constitutively expressed β1-4 galactosyltransferase (β4GalT1) that typically transfers UDP-galactose to terminal *N*-acetylglucosamine (GlcNAc) during glycoconjugate biosynthesis. However, in the presence of α-lactalbumin (described in detail below), also known as protein "B", which is expressed in response to lactation hormones, β4GalT1 shifts its acceptor specificity from GlcNAc to glucose, yielding lactose (Fig. 1B).[8]

For infants to benefit from lactose ingestion, the disaccharide must be hydrolyzed by lactase into its monosaccharide components, D-glucose and D-galactose. Lactase is produced in sufficient quantities to digest about 60–70 g of lactose daily in the small intestine. However, not all lactose from breast milk is absorbed by infants, a process known as physiological lactose malabsorption. This malabsorbed lactose is fermented in the colon to produce short chain fatty acids (SCFAs), hydrogen, carbon dioxide, methane, and lactic acid.[9,10] Commensal bacteria in the infant gut, particularly *Bifidobacteria* and *Lactobacilli*, preferentially salvage this leftover lactose, promoting the healthy development of the infant's microbiota.[11]

Lactose intolerance (LI) is a common gastrointestinal condition where the body does not have enough lactase to break down all consumed lactose. Roughly 70% of the world's population suffers from LI due to lactase non-persistence (LNP), a gradual decline in lactase expression after weaning.[9] Individuals who tolerate lactose beyond early childhood were most likely selected after the introduction of dairy farming and the growing consumption of cow's milk over 5000 years ago.[12] Fortunately, lactose intolerance is rare for children under five years of age; therefore, LI is an uncommon concern for breastfeeding mothers.

4.1.2 Oligosaccharides. Human milk oligosaccharides (HMOs) are the 4th most abundant molecule of human breast milk, with concentrations of *ca.* 20 g L^{-1} in colostrum and 5–15 g L^{-1} in mature milk.[13,14] Although present in the milk of most mammals, complex oligosaccharides are more diverse and abundant in primate milk. In humans, HMOs consist of five monosaccharides, D-glucose, D-galactose, L-fucose, GlcNAc, and *N*-acetylneuraminic acid (Fig. 2A). To date, over 200 HMOs have

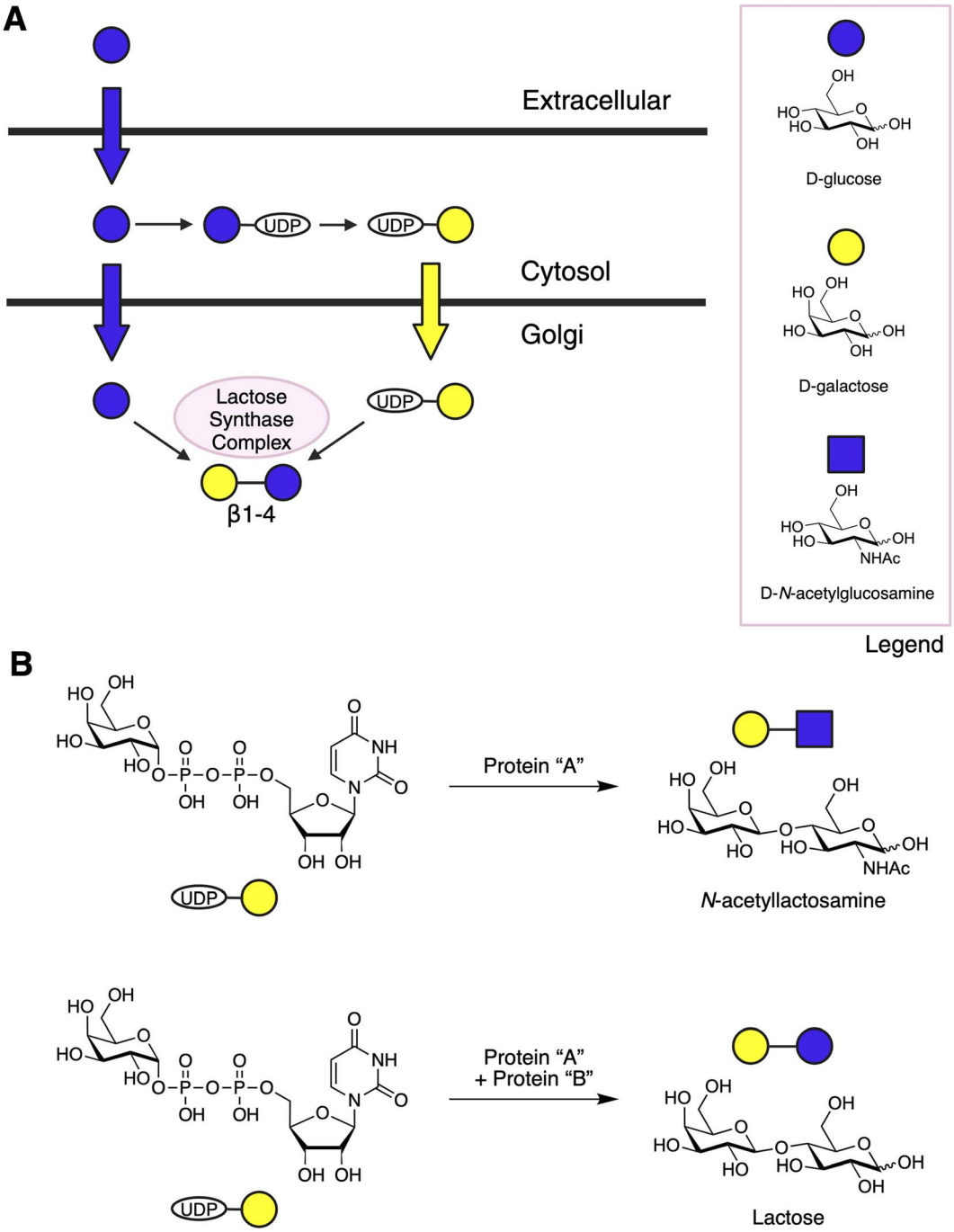

Fig. 1 Lactose biosynthesis in human milk. (A) Glucose and galactose are transported into the cytosol where glucose is converted to UDP-glucose then to UDP-galactose. UDP-galactose and glucose are then transported into the golgi where the lactose synthase complex facilitates their conjugation. (B) Protein "A" usually converts UDP-galactose to *N*-acetyllactosamine *via* addition of GlcNAc. However, in the presence of protein "B", protein "A" shifts its acceptor specificity from GlcNAc to glucose, yielding lactose.

been identified, with 2′-fucosyllactose (2′-FL) and 3-fucosyllactose (3-FL) being the first discovered in 1954.[15]

By the late 19th century, it was established that breastfed infants exhibited significantly greater resistance to disease compared to those fed bovine milk.[16] As the common structural feature of all HMOs is their lactose core, human mammary glands evolved mechanisms to diversify lactose biosynthetically *via* addition of lacto-*N*-biose (Galβ1-3GlcNAc-) or *N*-acetyllactosamine (Galβ1-4GlcNAc-) (Fig. 2B). Elongation *via* lacto-*N*-biose appears to terminate further chain growth, while elongation *via* *N*-acetyllactosamine permits continued chain extension. Lactose or the elongated oligosaccharides can be further diversified by addition of an L-fucose or *N*-acetylneuraminic acid residue (Figs. 2C–E). However, fucosylation is

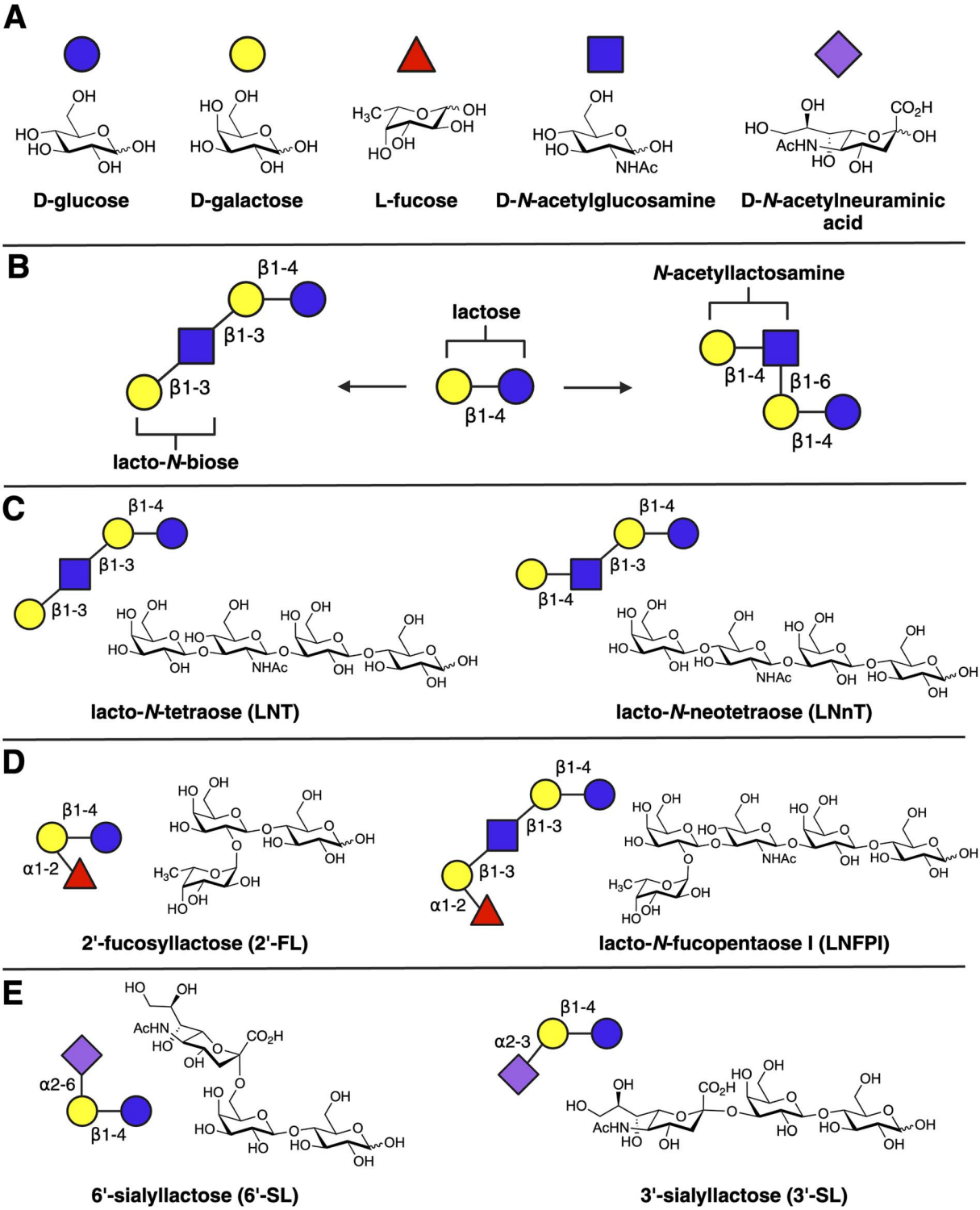

Fig. 2 HMO biosynthesis blueprint with representative HMO structures. (A) The five monosaccharides found in HMOs. (B) Lactose is the core of all HMOs and can be further extended *via* lacto-*N*-biose (β1-3) or *N*-acetyllactosamine. (C) Representative neutral HMOs. (D) Representative fucosylated HMOs. (E) Representative sialylated HMOs.

limited to α1-2, α1-3, or α1-4 linkages, while sialylation occurs in α2-3 or α2-6 linkages. Although the biosynthesis of lactose in mammary glands is known, the specificity of how lactose is extended to form HMOs remains poorly understood.

Across 89 publications, it was found that lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) are the most abundant neutral core HMOs (Fig. 2C), with percent abundances of 6.61% and 3.35%, respectively.[17] 2′-FL and lacto-*N*-fucopentaose I

(LNFPI) are the most abundant fucosylated HMOs (Fig. 2D) at 20.50% and 7.50% abundance, respectively. Finally, 6′-sialyllactose (6′-SL) and 3′-sialyllactose (3′-SL) are the most abundant sialylated HMOs with abundances of 3.63% and 1.71%, respectively. Putative compositions listed of the HMO fraction were based on g L^{-1}.

The oligosaccharide composition of breast milk is influenced by the mother's secretor status and blood group characteristics. Mothers with an active *Se* locus, which encodes for the α1-2-fucosyltransferase FUT2, are referred to as secretors. In contrast, nonsecretors lack FUT2, resulting in milk with limited levels of α1-2-fucosylated HMOs. However, *Se*-mothers can produce low amounts of α1-2-fucosylated HMOs, likely due to the activity of other FUT genes.[18] Individuals with an active *Le* locus, which encodes for the α1-4-fucosyltransferase FUT3, are classified as *Le* positive. These two loci create four distinct breast milk groups: *Se*+*Le*+, *Se*+*Le*−, *Se*−*Le*+, and *Se*−*Le*−, with typical frequency in the global population being 70%, 9%, 20%, and 1%, respectively.[17]

HMOs modulate the infant gut in various ways. First, commensal bacteria harbor the enzymes necessary to break down HMOs into their monosaccharide components to use as energy. This fermentation process generates short-chain fatty acids (SCFAs), which acidify the environment, promoting the growth of beneficial bacteria.[19] Further, SCFAs are associated with activation of the immune and inflammatory response.[20] HMOs also function as antiadhesive molecules through two mechanisms: by binding directly to pathogens or by binding to the intestinal epithelial cells, inducing conformational changes to their receptors.[21–23]

Our team and others have extensively reported on the antimicrobial activity of HMOs outside the infant gut against Group B *Streptococcus* (GBS).[24–26] Further, we have shown that HMOs disrupt biofilms formed by GBS, methicillin-resistant *Staphylococcus aureus*, and *Acinetobacter baumannii*.[27,28] HMOs have also been shown to interfere with the attachment of *Haemophilus influenzae* and *Streptococcus pneumoniae* to respiratory epithelium.[29] With extensive knowledge of the antimicrobial and antiadhesive properties of HMOs, future research will address characterizing their mechanism of action.

4.2 Fat

Fat is the 2nd largest component of human milk and plays a critical role in infant nutrition. Mechanistically, fats are critical to inflammatory responses, immune function, and cellular growth. Additionally, 50% of the child's energy supply originates from fat.[30] Mature milk contains *ca.* 40 g L^{-1} and there's triple the fat present in hindmilk than in foremilk.[31] The fats present in breast milk are readily digestible and absorbed due to the presence of bile salt-stimulated lipases that complement pancreatic lipases.[32] The major fats present in human milk are the fatty acid triglycerides, and two essential fatty acids linoleic acid (LA) and α-linolenic acid (Fig. 3).[33] LA and α-linolenic acid are precursors of arachidonic acid (ARA) and eicosapentaenoic

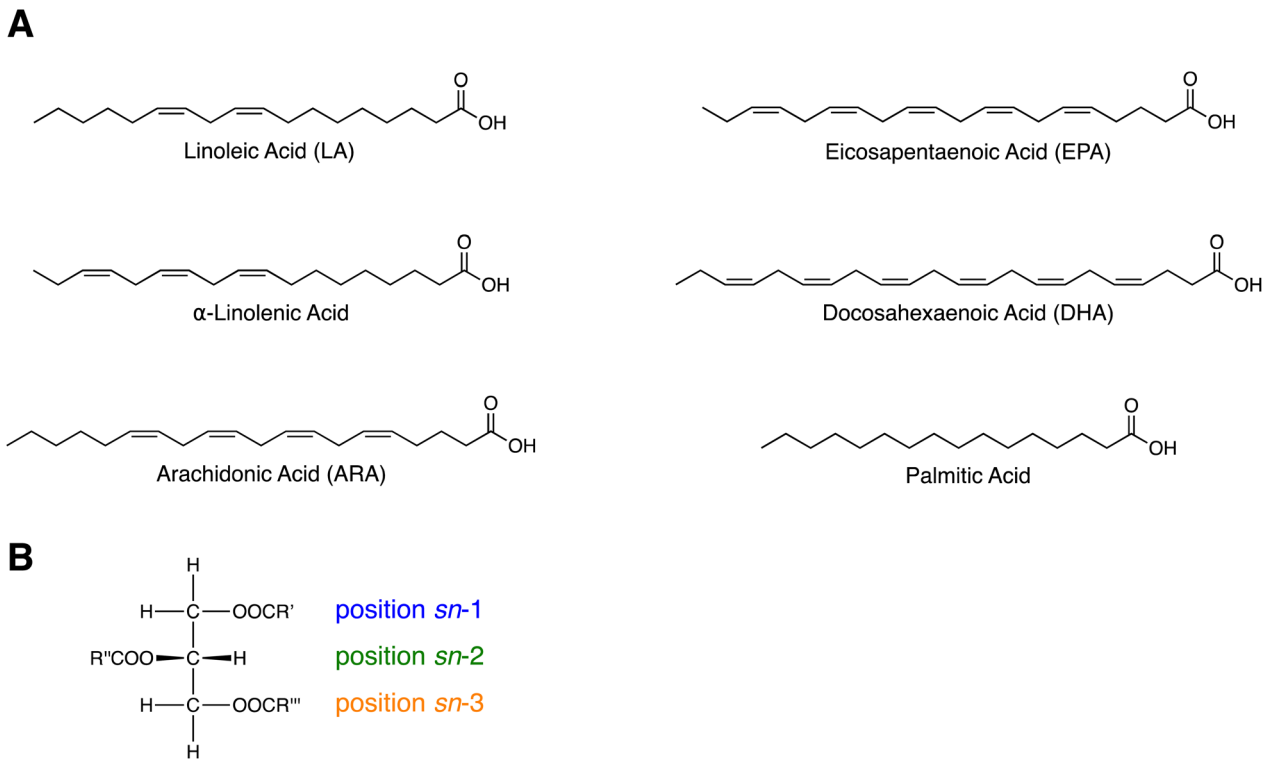

Fig. 3 Various fats present in human breast milk. (A) Structure of six fatty acids associated with human breast milk. This includes fatty acids directly present in breast milk as well as those synthesized from breast milk fatty acids. (B) Generic Fischer projection of a triglyceride to denote the *sn*-1, 2, and 3 positions.

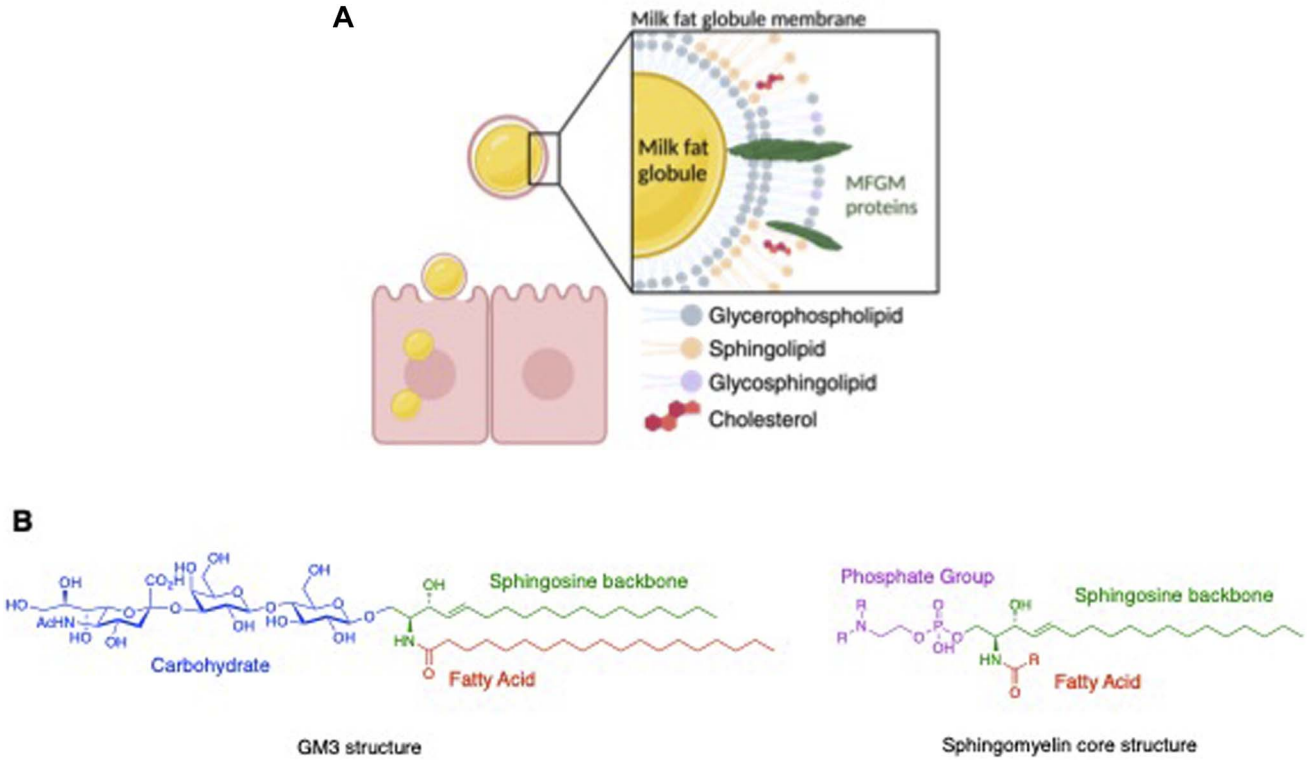

Fig. 4 MFGM delivers complex lipids and proteins to the infant. (A) Illustration of the milk fat globule membrane, which is a triple membrane structure comprising glycerophospholipids, sphingolipids, glycosphingolipids, cholesterol, and proteins. (B) Structures of GM3 ganglioside, the most common ganglioside in human milk, and sphingomyelin core found in breast milk. Ganglioside differentiation is in its carbohydrate portion. The R group in the sphingomyelin core refers to varying aliphatic chains.

acid (EPA), respectively. EPA can eventually be converted to docosahexaenoic acid (DHA) (Fig. 3).

Fats are important for inflammatory responses, immune function, and growth of the infant. In triglycerides, the *sn*-2 position is often occupied by palmitic acid (Fig. 3). The specific positioning of this fatty acid is critical as changing the position to *sn*-1 or *sn*-3 leads to impaired absorption of calcium and fat, negatively affecting bone accretion.[34] Further, palmitic acid at the *sn*-2 position is beneficial for intestinal and immunological health outcomes of the infant. In a mouse model for spontaneous enterocolitis, *sn*-2 palmitic acid supplementation resulted in decreased intestinal injury and inflammation.[35]

In the early postnatal period of preterm infants, there is a delicate balance of DHA, ARA, and LA levels. Indeed, decreased DHA levels are associated with an increased chance of chronic lung disease and decreased ARA levels are associated with an increased risk of late-onset sepsis.[36] Further, an increased LA : DHA ratio is associated with an increased risk of chronic lung disease and late-onset sepsis. Löfqvist found that low levels of ARA are also associated with an increased risk of retinopathy of prematurity, an eye disease occurring when abnormal blood vessels grow in the retina.[37] Various studies have investigated correlations between these polyunsaturated fatty acids and intestinal injury, inflammatory markers, and necrotizing enterocolitis (NEC), as recently reviewed by Martin.[38] Across these studies, it was found that *in vitro* and *in vivo* studies consistently demonstrate the anti-inflammatory actions of these fatty acids, yet more research is needed to draw conclusions about benefits of fatty acid-supplemented feedings against NEC.

Other complex lipids are delivered to the infant through milk fat globule membranes (MFGMs) and exosomes. MFGM is a triple membrane structure that encases milk fat and contains phospholipids, sphingolipids, cholesterol, and various proteins (Fig. 4A).[39] The delivery of MFGMs and exosomes also brings individual components, including sphingolipids, such as sphingomyelin and gangliosides (Fig. 4B), which modulate neonatal intestinal development, gut microbiota establishment, and inflammation.[40–43] Indeed, Blesso and his group observed that adult mice fed a diet containing 0.25% (wt/wt) milk sphingomyelin exhibited a lower abundance of gram-negative bacteria and a higher abundance of *Bifidobacteria* in their feces compared to those fed a high fat diet.[44] Similar results were found in preterm infants fed a ganglioside-supplemented diet compared to infants fed a control milk formula in which fecal contents of the ganglioside-supplemented group had lower amounts of *Escherichia coli* and higher amounts of *Bifidobacteria*.[45] This microbiota mediation could explain the decreased proinflammatory signaling in various models investigating ganglioside supplementation as *E. coli* is known to produce lipopolysaccharide (LPS), which triggers immune cells to release proinflammatory cytokines.[46–48]

Fat content in milk is closely related to maternal diet and weight gain during pregnancy. Interestingly, consumption of

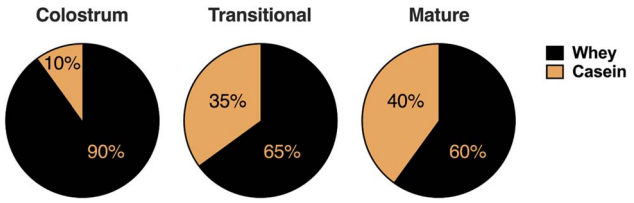

Fig. 5 Percentage of whey and casein type proteins at varying stages of lactation.

breads, snacks, fast foods, and margarines by lactating mothers can cause an increase in trans fatty acids in breast milk.[49] Trans fatty acid concentrations have adverse effects on infant growth and development and are inversely related to LA and α-linolenic acid.[50] ARA also correlates with ARA-rich food intake from lactating mothers, and EPA and DHA are also closely related.[51,52] Consequently, vegetarians have very low levels of DHA in their milk because of the lack of fish or other foods in their diet.[53] Therefore, it is recommended to take up to 300 mg of DHA per day to maintain enough DHA in breast milk.[54]

4.3 Protein

Human milk contains a diverse portfolio of proteins with various functions that contribute to the short- and long-term beneficial health outcomes of breastfeeding. The protein

Table 2 Approximate composition of whey proteins during stages of lactation in g L^{-1}. Adapted from Haschke *et al.*,[55] Lönnerdal *et al.*,[56] and Nagatomo *et al.*[57]

	Stage of lactation (days postpartum)			
Protein	Colostrum ~ (0–5)	Early ~ (6–15)	Transitional ~ (16–30)	Mature ~ (30–360)
α-Lactalbumin	4.56	4.3	3.52	2.85
Lactoferrin	6.15	3.65	2.46	1.76
Osteopontin	0.18	1.49	—	0.138
Secretory IgA	5.45	1.5	1.10	0.138
Lysozyme	0.32	0.30	0.28	0.38
Totals	20.6	15.7	14.8	11.1

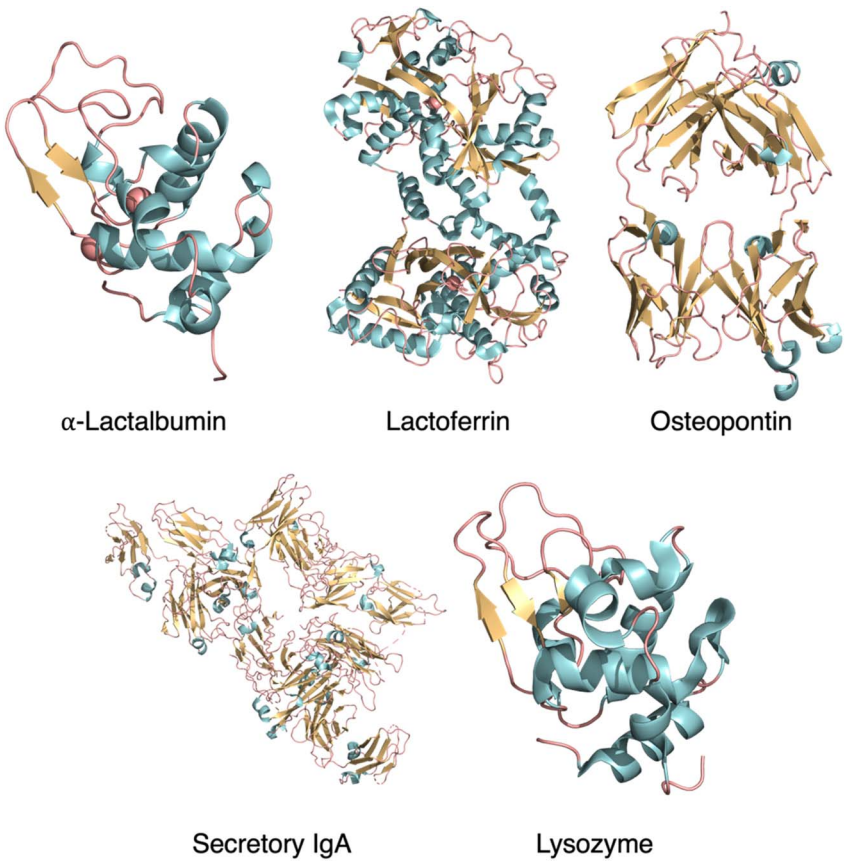

Fig. 6 PDB structures of α-lactalbumin,[109] lactoferrin,[110] osteopontin,[111] secretory IgA,[112] and lysozyme.[113] Helices shown in blue, β-sheets shown in orange, loops shown in pink.

content of milk at birth is *ca.* 20 g L^{-1} and decreases to *ca.* 11 g L^{-1} at six months postpartum. Human milk proteins can be classified into three categories: whey, casein, and mucin, however, mucin is only present in the milk fat globule membrane (MFGM). Human milk is whey dominant, yet the whey : casein ratio fluctuates during stages of lactation (Fig. 5). Certain proteins facilitate infant development by providing amino acids, stimulating intestinal growth and maturation, governing the composition of the microbiome, and enhancing learning. Other proteins are present to improve the bioavailability of vitamins and minerals. Lastly, there are proteins that provide defense against pathogens.

4.3.1 Whey. Proteins in human milk are mainly whey proteins (60–90%) – the highest concentration of which include α-lactalbumin, lactoferrin (LF), osteopontin (OPN), secretory IgA, and lysozyme (Table 2 and Fig. 6).

4.3.1.1 α-Lactalbumin. α-Lactalbumin is a small, acidic, Ca^{2+} binding protein that constitutes about 25–35% of the total protein of human breast milk.[58–61] Concentrations of α-lactalbumin range from 2-3 g L^{-1} in breast milk. Structurally, α-lactalbumin is a single-chain polypeptide of 123 amino acids, with high levels of tryptophan, lysine, and cysteine, providing a well-balanced supply of essential amino acids for the growing infant.[58] Clinical studies have demonstrated that increasing the proportion of α-lactalbumin to other breast milk proteins results in plasma levels of essential amino acids comparable to those found in breastfed infants.[62] Further, Hernell's group found in a double-blind randomized control trial that infants fed an α-lactalbumin-enriched formula exhibited growth patterns similar to breastfed infants.[63] Similar findings were reported in a study comparing age-appropriate growth, head circumference, and plasma essential amino acids concentrations, where infants receiving α-lactalbumin-enriched formula showed growth outcomes more comparable to breastfed infants than those fed standard formula.[64]

The metal binding-capabilities of α-lactalbumin facilitate enhanced mineral absorption in infants, which is crucial for proper growth and development. In its unbound state, α-lactalbumin exists in the molten globule-like state, but the binding of metals such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, and K^{+}, stabilizes its structure.[59,65] α-Lactalbumin is hypothesized to bind various metals due to its structural flexibility in its molten globule-like state and presence of coordinating amino acid residues.

α-Lactalbumin plays a pivotal role in breast milk as it serves as the "B" component of the two-protein lactase synthase complex in the golgi of mammary cells (Fig. 1). During milk production, α-lactalbumin is transported from the inner surface of the golgi to mammary secretory vesicles and finally to the alveolar lumen. In the lactose synthase complex, α-lactalbumin interacts with β1-4-galactosyltransferase (β4GalT1), the "A" component, which typically transfers galactose to terminal GlcNAc residues. Yet, in the presence of α-lactalbumin, β4GalT1's substrate specificity shifts almost exclusively to glucose, enabling the synthesis of lactose.[66] Once α-lactalbumin binds β4GalT1, a large conformational change occurs in the sugar-binding region of the enzyme. This reorganization causes a difference in the hydrophobic pocket where GlcNAc usually binds, limiting such an interaction. Further, α-lactalbumin directly stabilizes glucose *via* binding its O1 hydroxyl group, increasing binding efficiency. α-Lactalbumin's interaction with β4GalT1 lowers the K_m for glucose by 1000-fold, significantly enhancing the production of lactose over other disaccharides.[67]

4.3.1.2 Lactoferrin. Lactoferrin (LF) is an 80 kDa glycoprotein that exists in two primary isoforms: an apo-form (iron-free) and a holo-form (iron-bound). The concentration of LF in milk is highest in colostrum at *ca.* 6 g L^{-1} and drops to *ca.* 3 g L^{-1} after a month of lactation.[56,68] Numerous studies have highlighted the antimicrobial and immunomodulatory activates of LF. LF exhibits antimicrobial effects against viral, fungal, parasitic, and bacterial pathogens.[69,70] This activity often extends from its iron-binding abilities where the chelation of this essential nutrient starves invading microorganisms; a process termed "nutritional immunity".[71–73] Beyond its iron-chelating role, LF also exerts direct antimicrobial effects by binding to bacterial cell wells causing destabilization.[74–76]

A randomized control trial in humans showed that bovine LF supplementation, either alone or in combination with *Lactobacillus rhamnosus* GG decreased late onset sepsis very low birth weight (VLBW) neonates.[77] A subsequent study further demonstrated that bovine LF prevented invasive fungal infections in VLBW infants.[78] High levels of fecal LF in neonates were positively correlated with the establishment of the gut microbiota, illustrating LF's key role in beneficial organism habitation.[79] Additionally, a hydration drink containing 1.0 g L^{-1} recombinant human LF and 0.2 g L^{-1} lysozyme reduced the duration of diarrhea in 5- to 35- month-olds hospitalized for diarrhea in Peru.[80]

LF is also critical for maintaining immune homeostasis, serving as a bridge between innate and adaptive immunity through modulation of antigen-presenting cells (APCs) like macrophages, dendritic cells, and B cells.[81] In macrophages, LF enhances antigen presentation while reducing excessive inflammation. LF also aids dendritic cell maturation and migration, boosts B cell antibody production, and helps balance pro- and anti-inflammatory actions in T cells. In piglets, bovine LF intake modulated immune development by activating a balanced T-helper-1/2 cytokine response as reviewed by Donovan.[82] Together, LF is critical for microbiome maturation.

4.3.1.3 Osteopontin. Osteopontin (OPN) is a multifunctional bioactive protein with a concentration of *ca.* 0.140 g L^{-1} in human milk,[83] though levels vary depending on the stage of lactation and the mother's geographic location.[84] This 314-amino acid protein is negatively charged, glycosylated, and highly phosphorylated.[85] OPN is critical in the development of the infant's immune system by influencing the function of macrophages, dendritic cells, and T cells.[86,87] Notably, OPN is an early regulator of T helper 1 cell-mediated immunity by activating IL-12 secretion and inhibiting IL-10 production.[88] OPN also enhances host resistance to infection and facilitates phagocytosis by binding to bacterial pathogens, making them more recognizable by phagocytes.[89–91]

Donovan's group investigated the effects of bovine OPN in infant rhesus monkeys. In this study, the control group was fed a standard milk-based formula, while the test groups received

either a formula containing 0.125 g L^{-1} bovine milk OPN or were nursed by their mothers.[92] When comparing intestinal transcriptomes, they found that the formula supplemented with OPN resulted in only 217 differently expressed genes (DEGs) compared to the nursed monkeys', whereas the standard formula led to 1017 DEGs. This suggests that adding OPN to formula shifted the gene expression closer to that of nursed monkeys. Another study by Sørensen explored the effects of OPN on intestinal Caco-2 cells.[93] They found that bovine and human milk OPN activated the expression of 322 and 239 genes, respectively. Analyzing 131 genes that were similarly expressed in response to both human and bovine OPN revealed that biological processes related to the ubiquitin system, DNA binding, transcription, and translation were impacted by OPN. Collectively, these studies demonstrate OPN's ability to modulate gene expression in the infant gut.

4.3.1.4 Secretory immunoglobulin A. Secretory immunoglobulin A (SIgA) is the most abundant immunoglobulin in human milk, with its highest concentration found in colostrum at *ca.* 2.5 g L^{-1}, decreasing to *ca.* 1 g L^{-1} between days 8–12, and stabilizing to *ca.* 0.7 g L^{-1} in mature milk.[94] SIgA consists of two IgA monomers linked by a J chain, and during its translocation across the epithelium, it acquires the secretory component from the polymeric Ig receptor. There are two forms of SIgA: a T-cell dependent version that is monoclonal and forms high-affinity interactions, and a T-cell independent form, which is polyclonal and binds with lower affinity.[95,96]

SIgA plays a crucial role in shaping the microbiota by interacting with both pathogenic and commensal organisms. Against pathogens, SIgA facilitates the clumping of bacteria, which are then moved through and out of the intestine.[97] SIgA also prevents the translocation of pathogens across the intestinal epithelium and promotes the colonization of symbiotic bacteria by supporting their biofilm formation.[98,99] In this vein, several commensal organisms such as *Bacteroides fragilis* and members of *Lachnospiraceae* have evolved to increase SIgA binding and enhance their colonization. Other probiotics including *Bifidobacterium* and *Enterobacteriaceae* are abundant in IgA+ fractions obtained from feces, which explains their early establishment of the infant microbiota.[100] How SIgA selectively induces clumping and removal of pathogens *versus* promoting colonization of commensals remains unclear.

By promoting the colonization of beneficial microorganisms in the maturing infant gut, SIgA also contributes to immune function. Evidence also exists demonstrating SIgA's immune dampening abilities, which promotes a more regulated immune response as the infant matures.[101,102] As weaning occurs and the external source of SIgA decreases, the infant's body compensates by producing its own SIgA *via* active intestinal transport,[103] underscoring the antibody's role in immune defense and microbiota development during early growth.

4.3.1.5 Lysozyme. Lysozyme is a 15 kDa protein responsible for lysing bacteria by hydrolyzing the β1-4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid monosaccharides in peptidoglycan, a key component of bacterial cell walls.[104] The concentration of lysozyme in human milk varies, ranging from *ca.* 0.37 g L^{-1} in colostrum, *ca.* 0.27 g L^{-1} in transitional milk, and *ca.* 0.24–0.89 g L^{-1} in mature milk.[105] As lysozyme disrupts bacterial cell walls, leading to cell death, it is believed to selectively target pathogenic organisms. Several organisms have evolved mechanisms of resistance to lysozyme, such as peptidoglycan modifications. Yet, lysozyme has also been shown to activate the pro-inflammatory immune response, which is designed to target pathogenic organisms.[106] Indeed, a study by Elizabeth Maga's group found that six-week-old pigs fed goat milk enriched with lysozyme had higher abundance of bacteria associated with positive gut health (*Bifidobacteriaceae* and *Lactobacillaceae*) and a lower abundance of bacteria associated with disease (*Mycobacteriaceae*, *Streptococcaceae*, and *Campylobacterales*).[107] Further, they showed that lysozyme can modulate the inflammatory response in the gut. Pigs fed the human lysozyme-enriched goat milk had a significantly higher level of the anti-inflammatory cytokine TGF-β1 in a portion of their small intestine, without an increase in proinflammatory cytokines.[108] Together, lysozyme's dual function of selectively eliminating pathogenic bacteria while modulating the immune system helps protect the infant from infections, supporting the development of a healthy microbiota.

4.3.2 Casein. Milk casein typically exists in the form of micelles, with an average size of 40–100 nm.[114] These micelles consist of three main components; β-casein and α-S1-casein, which form the inner core, and κ-casein, which makes up the glycosylated outer layer that stabilizes the micelle.[115] During the first year of lactation, the concentrations of β-casein, α-S1-casein, and κ-casein range from 0.04–4.42 g L^{-1}, 0.04–1.68 g L^{-1}, and 0.10–1.72 g L^{-1}, respectively[116] (Table 3).

Functionally, casein provides essential amino acids to the infant. Casein's ability to bind and transport divalent cations, such as zinc and calcium, suggests it may also aid in the absorption of these nutrients, although more research is needed to confirm this activity.[117,118] With heavy glycosylation, κ-casein has been shown to bind pathogens, including *Helicobacter pylori*, thereby preventing attachment and infection.[119] In 2023, Szeto and Zhao found that κ-casein was positively correlated with *Clostridium butyricum*, an intestinal symbiotic bacterium that has a strong butyric acid production capacity.[120] This SCFA reduces the production of inflammatory cytokines by reducing the permeability of the intestinal epithelium, which is crucial for early intestinal immune system development.[121] Casein is useful in breast milk as an antimicrobial and a provider of essential amino acids.

Table 3 Approximate composition of casein proteins during stages of lactation in g L^{-1}. Adapted from Lönnderdal *et al.*[116]

	Stage of lactation (days postpartum)		
Protein	Early ~ (0–10)	Transitional ~ (11–30)	Mature ~ (30–360)
β-casein	1.29	1.46	1.03
α-S1-casein	0.34	0.33	0.33
κ-casein	0.86	0.80	0.55
Totals	2.49	2.59	1.92

4.3.3 Mucin. Mucin is often overlooked as a significant component of the total protein in breast milk due to its location in the milk fat globule membrane (MFGM, Fig. 4A).[56] However, for the purposes of this review, mucin's roles as a protein merits attention. The mucin concentration in human breast milk ranges from 0.654–0.804 g L^{-1}.[122]

Mucins serve multiple functions, but are best known for protecting infants from infection, specifically *via* their glycosylated structures. Interestingly, most infants who are exclusively breastfed by human immunodeficiency virus (HIV)-positive mothers remain uninfected even after repeated exposure through breastmilk.[123] Mucins have been implicated in this phenomenon with Saeland illustrating that MUC1 blocks infection by HIV-1.[124] During mother-to-child transmission of HIV, the virus typically targets dendritic cells in mucosal areas, facilitating transmission to T-cells. However, O-linked glycans on MUC1 efficiently bind dendritic cell receptors, thereby blocking HIV-1 transmission. Additionally, mucins inhibit viral replication by binding to rotavirus and Norwalk virus.[125,126]

Mucins are also effective against bacterial colonization. MUC1 and MUC4 have been shown to block *Salmonella enterica* serovar typhimurium invasion of Caco-2 cells.[127] MUC1 was also shown to provide a protective barrier against *H. pylori* colonization in a mouse model.[128] Together, the mucins found in MFGM serve to protect the developing microbiota from unwanted habitation by viral and bacterial pathogens.

4.4 Hormones and growth factors

Human breast milk contains various biologically active factors such as hormones, peptide growth factors, and cytokines (Fig. 7). One such hormone, erythropoietin (epo), is responsible for increasing red blood cells, which may prevent premature anemia.[129,130] Epo is also critical for tightening intestinal junctions in infants, protecting the child from any milk-borne pathogens or viruses.[131] Further, epo helps maintain the integrity of the mammary epithelium, preventing HIV particles from seeping from the blood into milk.[132]

Calcitonin is a growth-regulating hormone present in human milk. Serum levels of calcitonin are higher in pregnant and lactating women as compared to non-pregnant women and are not influenced by breastfeeding.[133,134] Therefore, it is assumed that these increased levels are to protect the healthy maternal skeleton by opposing the action of 1,25-dihhydroxycholecalciferol ($1,25[OH]_2D_3$), a bone-resorbing hormone that exhibits increased levels during pregnancy as the mother needs excess calcium.[133] In the context of infant health, calcitonin has been proposed to play a role in the maturation of enteric neurons.[135]

Human breast milk also contains adiponectin and leptin, which are adipose-derived hormones that regulate metabolism and body composition. Adiponectin promotes tissue insulin sensitivity, stimulates glucose uptake, and decreases energy expenditure.[136,137] Leptin helps regulate appetite by informing the infant's brain of remaining energy sources.[138,139] While some studies suggest a link between these hormones and a reduced risk of obesity, results have been inconsistent.[140–143] These

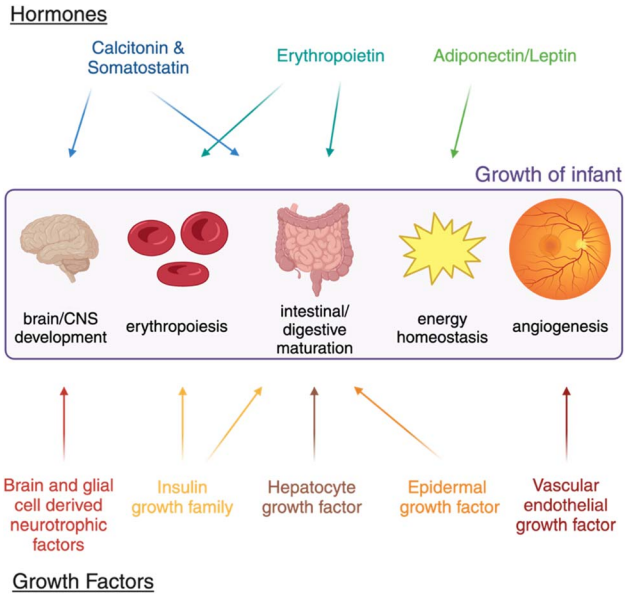

Fig. 7 A representative schematic showing the distribution of hormones and growth factors found in breast milk and what they provide to the growing infant.

variations may stem from differences in study methodologies, breast milk composition, and other health-related factors.

Epidermal growth factor (EGF) plays a vital role in the maturation and healing processes of the intestinal mucosa. EGF levels in human breast milk are highest in the first days after birth at roughly 100 ng mL^{-1} and then gradually decrease during lactation.[144,145] EGF is resilient against low pH and digestive enzymes, allowing it to freely reach the intestines where it stimulates DNA synthesis, cell division, protein synthesis, and the absorption of critical nutrients.[146,147] EGF also heals damaged mucosa by correcting alterations in tight junction proteins induced by the proinflammatory cytokine TNF-α.[148,149] Heparin-binding EGF (HB-EGF), a member of the EGF family, is primarily responsible for the protection of the intestinal epithelium from hypoxic necrosis and cytokine-induced apoptosis.[150–154] HB-EGF provides these functions mainly by decreasing nitrogen and oxygen reactive species production.[155]

Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are two of the most abundant growth factors in breast milk, especially in colostrum.[156,157] VEGF protects against angiogenesis and vasculogenesis, promotes growth, and protects the infant's gastrointestinal system.[158–161] Similarly, HGF stimulates epithelial cell proliferation and also contributes to tissue regeneration, defense, and longevity.[162–164]

The insulin-like growth factor (IGF) family, specifically IGF-I and IGF-II, is found in its highest levels in the colostrum.[165,166] This family enhances cell survival, stimulates the proliferation of intestinal stem cells, and promotes erythropoiesis.[167–169] In rat models, IGF-I protects against necrotizing enterocolitis (NEC) and reduces the inflammatory response.[170] One study by van Goudoever's group found that adding double the usual amount of IGF-I in colostrum improved gut barrier function by

day 14.[171] Finally, both IGF-I and -II were shown to promote cell migration of human umbilical cord vascular endothelial cells, which protects and develops the maturing intestinal epithelium.[172,173]

Proper nervous system development requires both brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). BDNF plays a role in nervous system development and maintenance and has been implicated in the modulation of learning and memory.[174] Additionally, BDNF regulates enteric neuronal activity, increasing gastrointestinal motility.[175] GDNF is critical for neuronal survival in the enteric nervous system.[176,177] Together, BDNF and GDNF stimulate neurodevelopment of the infant during breastfeeding.

5 Outlook

Ontology. Despite its importance in advancing infant health and wellness, there are major challenges preventing our complete understanding of human milk. Currently, we understand milk to be a personally curated collection of key nutrients, macromolecules, and microorganisms used by the infant. However, we understand little about the ontology of human milk, that is, the mechanistic interplay between the components of the milk and how they complement each other to correctly guide growth and development. In a related vein, we have a poor understanding of the feedback loop between mother and child that governs *how* and *when* components change. Key gaps in this area include characterizing variability in human milk composition based on environmental determinants of health and evaluating effects of maternal nutrient supplementation during lactation on human milk composition.

Defining milk quality. Given the large number of complex molecules present in milk, the greatest key gap in analysis is that there is no single analytical technique capable of all aspects of milk analysis. Mass spectrometry is the most enabling tool to characterize the composition of human milk. New technologies, particularly those capable of separating molecules with near identical polarity, such as HMOs, would be a great advance toward defining global reference values for macromolecules, micronutrients, and microorganisms in human milk.

Microbial metabolites. Microorganisms and their metabolites are increasingly being used as therapeutics. Even though human milk has a robust microbiome, to date, characterization of microbial metabolites in human milk, and their impact on the infant's health is incomplete. This is a key gap as these metabolites may potentiate developmental, metabolic, or immune programming in the child. Breast milk microbiome-associated metabolites are an intriguing avenue for new therapies. It would be informative to characterize which metabolites produced by the various microbiota of the mother are transported to the mammary gland during lactation. Moreover, it is unclear whether levels of diet-derived maternal microbial metabolites in human milk can be modulated by dietary intervention or by supplementation. Taken together, maternal and microbial metabolites represent promising targets for 'postbiotic' intervention during nursing. Given the current state of analytical chemistry, it is possible to use multi-omic approaches, data science, and statistical analysis to characterize this facet of human physiology.

After water, human milk is the 2nd most important liquid in existence. Yet, many unknowns remain. In a way, milk is an example of precision nutrition – its components unambiguously determined and produced by the mother specifically for her infant. It is undefined, however, how this occurs. We hypothesize that the retrograde flux that occurs during nursing is a channel for bidirectional signaling or the exchange of metabolites between the infant's mouth and the mammary gland. We recognize, however, that the composition of mother's milk may also be the result of environmental and genetic factors. Taken together, human milk science provides exciting opportunities to improve maternal–child health using frontier areas such as artificial intelligence, synthetic biology, machine learning, and advanced mass spectrometry. The ultimate deliverable of addressing these key gaps is characterizing new maternal and child nutrition interventions that may inform the politics of health policies.

6 Data availability

As this is a review article, there is no new data associated with this manuscript.

7 Conflicts of interest

There are no conflicts to declare.

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