

Building a Foundation

NEW INSIGHTS

INTO PEDIATRIC NUTRITION: MILK FAT GLOBULE MEMBRANE

Insights From Human
Milk Research

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Origin and Structure
of MFGM

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Scientific Evidence for
Health Benefits of MFGM

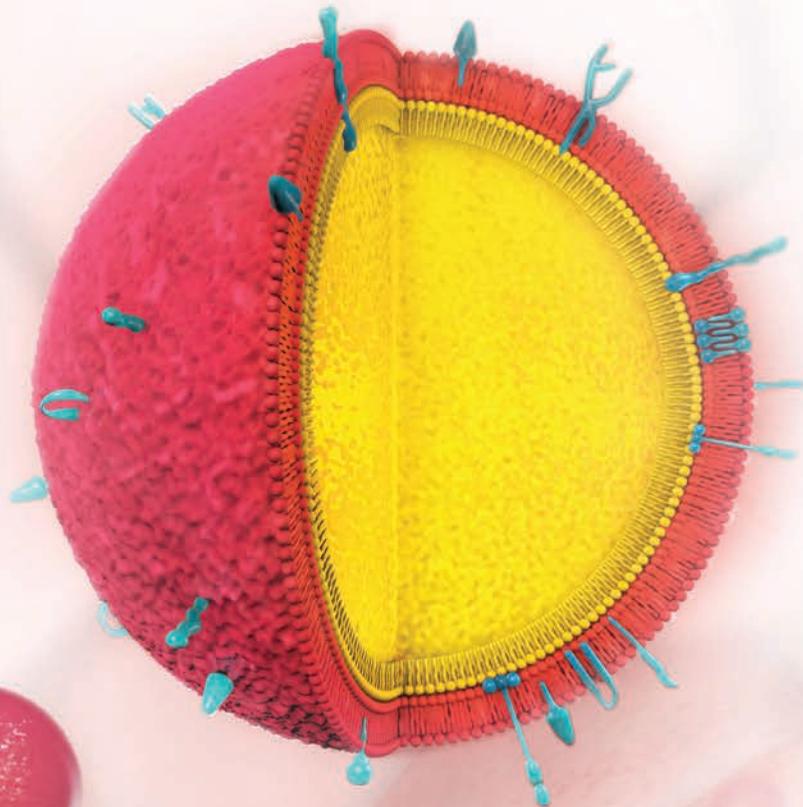
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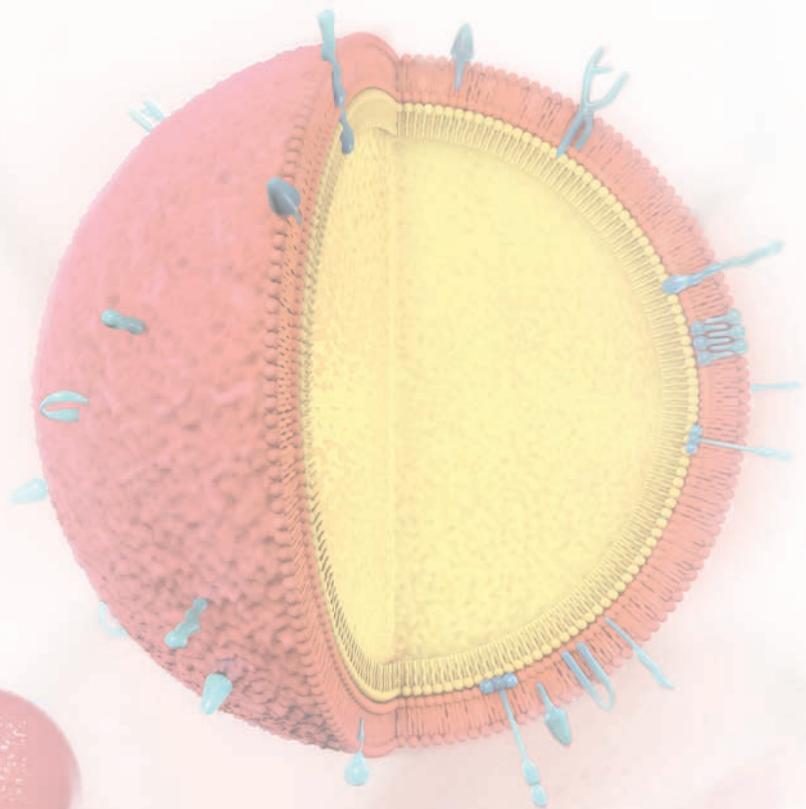


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Introduction

A. Nutrition and Early Infant Development

Infancy and early childhood represent a critical period of time for nutrition, as proper dietary intake during this phase is vital to supporting not only early growth and development, but also optimal health outcomes later in life.¹ Nutritional and environmental influences are especially important during the first 2-3 years of life, when growth is the most rapid. Weight doubles in the first 4-6 months of life, and triples in the first year (to 10 kg or 22 lbs).² Length increases by 50% at one year, and 75% at two years.² This is also a period of rapid development of the nervous system; indeed, the brain nearly triples in size in the first two years, reaching approximately 85% of adult weight.^{3,4} Also crucial to early development is an appropriate balance of nutrients—protein, carbohydrate, lipid, and micronutrients—the requirements for which change significantly over the course of infancy and childhood. For instance, infants and toddlers need a high proportion of dietary fat (up to 50% of total calories in the first two years) in order to support rapid myelination; these needs change as nervous system development slows throughout later childhood and into adolescence.⁵ Therefore, optimal nutrition during infancy must be targeted to the unique needs of the growing infant at this stage—and is crucial for young children to reach their full potential in growth, health, and cognitive development later in life.¹

The challenge of optimizing nutrition, therefore, is to identify and promote a diet containing an appropriate blend of nutrients that best supports healthy growth and development at each stage of life. Since the ideal source for infant nutrition is healthy maternal milk,⁶ a robust understanding of human milk composition and function is essential in order to develop the best nutritional strategies for all infants, whether breast-fed or formula-fed. The composition of human milk can vary

... the ideal source for infant nutrition is healthy maternal milk⁶ ...

across individuals, populations, environmental settings, and phases of lactation;⁷⁻⁹ therefore, understanding the needs of a broad range of infants is important for designing proper nutritional support. At the same time, many fundamental aspects of human milk composition are shared in common across populations; for example, a fat caloric content of approximately 50% in order to support the needs of rapid growth and neural development in infants.^{6,10}

B. Insights From Human Milk Research

Research into the composition and functionality of human milk over the past several decades has provided valuable insights into the nutritional needs of infants, as well as inspired innovations in the design of infant formula to bring it compositionally closer to human milk. One example of such innovation to the carbohydrate content of infant formula is the addition of prebiotic oligosaccharides, which have been demonstrated to support development of the gut immune system.¹¹ Another example of infant formula innovation is the addition of the long chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), present in human milk but originally unsupplemented in infant formula. Following many years of research into the safety and benefits of these LCPUFAs

to health, cognition, and visual development, DHA is now widely recognized to be important in the diets of infants and young children during the first 24 months of life.^{12,13}

Recent and ongoing research continues to expand our understanding of other important, but previously under-recognized, functional components in human milk. A number of national and international cohort studies have used state-of-the-art approaches to generate new biological insights into human milk composition, including the function of various proteins, oligosaccharides, fatty acids, vitamins, and minerals.^{8,9,14} Advanced techniques including mass spectrometry, bioinformatics, and proteomics allow researchers to examine and identify even minor components present in human milk. Such insights not only advance the understanding of infant health and development, but also provide a basis for further innovation and improvement in the design of infant formula. At the same time, research into functionality and outcomes of milk components has highlighted the concept that striving to approximate the functional benefits of human milk, and not simply the composition, is of paramount importance.

Nutrients in human milk, as throughout the infant diet, perform three major distinct roles: They may be sources of energy, provide structural building blocks, or act as functional or bioactive compounds. The latter can be defined as constituents that affect biological processes and thus have an impact on body function or condition and ultimately health, beyond basic nutrition.^{7,15} Bioactive components in human milk represent a large and complex category which includes proteins, oligosaccharides, fatty acids, growth factors, hormones, and even intact cells; these are derived from a variety of sources, and have diverse functions and potential health benefits.⁶ Some nutrients can potentially fulfill more than one role; for instance, lactose is not only a primary carbohydrate energy source, but also functions as a potential prebiotic, as some dietary lactose may reach the colon undigested and thereby influence the development of

gut bacterial flora.^{16,17} Furthermore, research has elucidated additional complexities of human milk nutrient structure, which reflect the lactation process itself. A particular example is the secretion by mammary epithelial cells of the milk fat globule, which carries with it into milk a diverse collection of membrane-bound proteins, carbohydrates, and lipids transferred directly from the surface of maternal cells.⁷

Therefore, the purpose of this monograph is to provide an overview of this structurally complex bioactive milk component, the milk fat globule membrane (MFGM). It will review the structure and physiology of MFGM, highlight over two decades of foundational research into MFGM and its components, and examine the body of evidence supporting the potential benefits of MFGM in preclinical models as well as in the diet of infants and young children.

What Is the Milk Fat Globule Membrane (MFGM)?

A. Origin of MFGM

Milk lipids are secreted in a unique manner by lactocytes, specialized epithelial cells within the alveoli of the lactating mammary gland. First, fat synthesized within the endoplasmic reticulum accumulates in droplets between the inner and outer phospholipid monolayers of the endoplasmic reticulum membrane. As these droplets increase in size, the two monolayers separate further and eventually pinch off, surrounding the droplet in a phospholipid monolayer vesicle that allows it to disperse within the aqueous cytoplasm. Lipid droplets then migrate to the apical surface of the cell, where plasma membrane subsequently envelops the droplet and extrudes together with it, fully encasing the fat droplet in an additional bilayer of phospholipids. The milk fat globule thus released into the glandular lumen, measuring 3-6 μm in average diameter, is surrounded by a phospholipid trilayer containing associated proteins, carbohydrates, and lipids derived primarily from the membrane of the secreting lactocyte. This trilayer is collectively known as MFGM (Figure 1).^{18,19} While MFGM only makes up an estimated 2% to 6% of the total milk fat globule,¹⁸ it is an especially rich phospholipid source, accounting for 60% to 70% of total milk phospholipids.^{20,21} In contrast, the inner core of the milk fat globule is composed predominantly of triacylglycerols, containing long-chain fatty acids such as DHA and ARA.

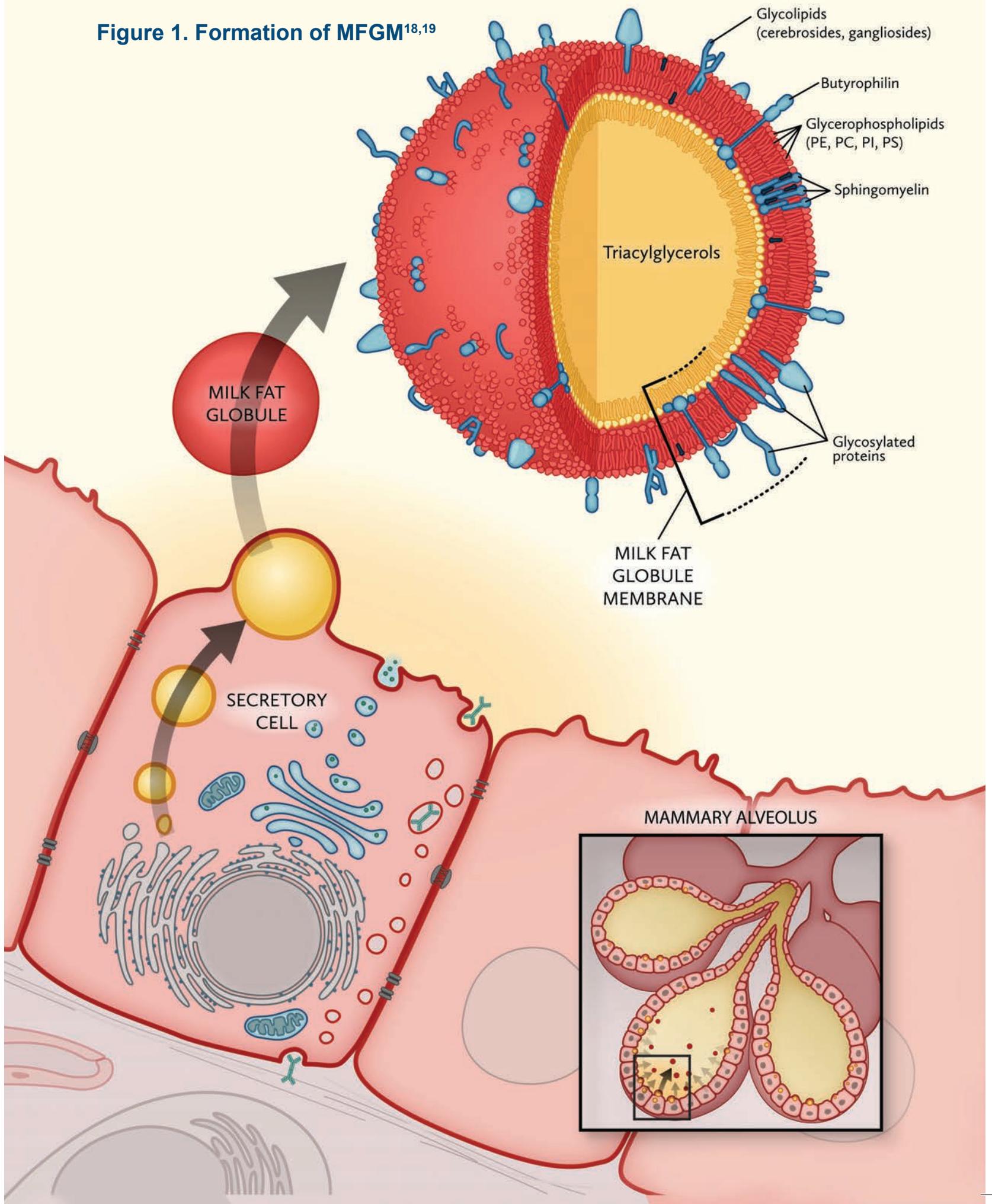
This type of secretion process occurs in all types of mammalian milk, including human and bovine, and is distinct from the lipid secretion mechanism used by all other non-mammary cells; thus, MFGM is unique to milk and is not present in non-dairy food products.¹⁹ Furthermore, genes associated with the production of the milk fat globule and membrane appear to be the most conserved lactation genes throughout evolution, consistent with a significant physiologic role for the MFGM

structure.²² Although many of the compounds within MFGM are identified as bioactive, and have been linked to cognitive and health benefits as discussed below, indeterminate amounts of these components have traditionally been lost during commercial dairy processing. Non-dairy fat sources, such as vegetable oils, also contain variable amounts of phospholipids and sphingolipids.²³ However, more recent advances in technology have facilitated the separation of MFGM from the fat globule, allowing bovine MFGM to be added in concentrated form.²⁴ Although differences have been reported, bovine MFGM generally contains a lipid and protein composition which is similar to that of human MFGM.^{25,26} Thus, the enrichment of infant formula with these fractions represents an opportunity to more closely match the composition and functionality of breast milk, and thereby to provide potential health benefits for formula-fed infants.

B. Structure and Components of MFGM

The MFGM structure is complex and comprises a variety of phospholipids, glycolipids, proteins, and glycoproteins, along with cholesterol and other lipids. Specific lipids and proteins are localized to different layers of the membrane, with carbohydrate chains of glycoproteins and glycolipids directed toward the outer surface of the milk fat globule; the lipid-to-protein weight ratio in MFGM is approximately 1:1.²⁷ However, the nutritional significance of these components is defined not only by their structure or macronutrient category, but also by the physiological role that each nutrient serves. As a quantitatively minor presence within milk, MFGM likely contributes little to energy production, but its constituents may confer structural and functional benefits (Figure 2).^{18,24} Many of these nutrients are known to play important functional roles within the gut, brain, and elsewhere in the body; the functions of other components are still being elucidated. Key individual components of MFGM are highlighted in the sections below.

Figure 1. Formation of MFGM^{18,19}



Lipids

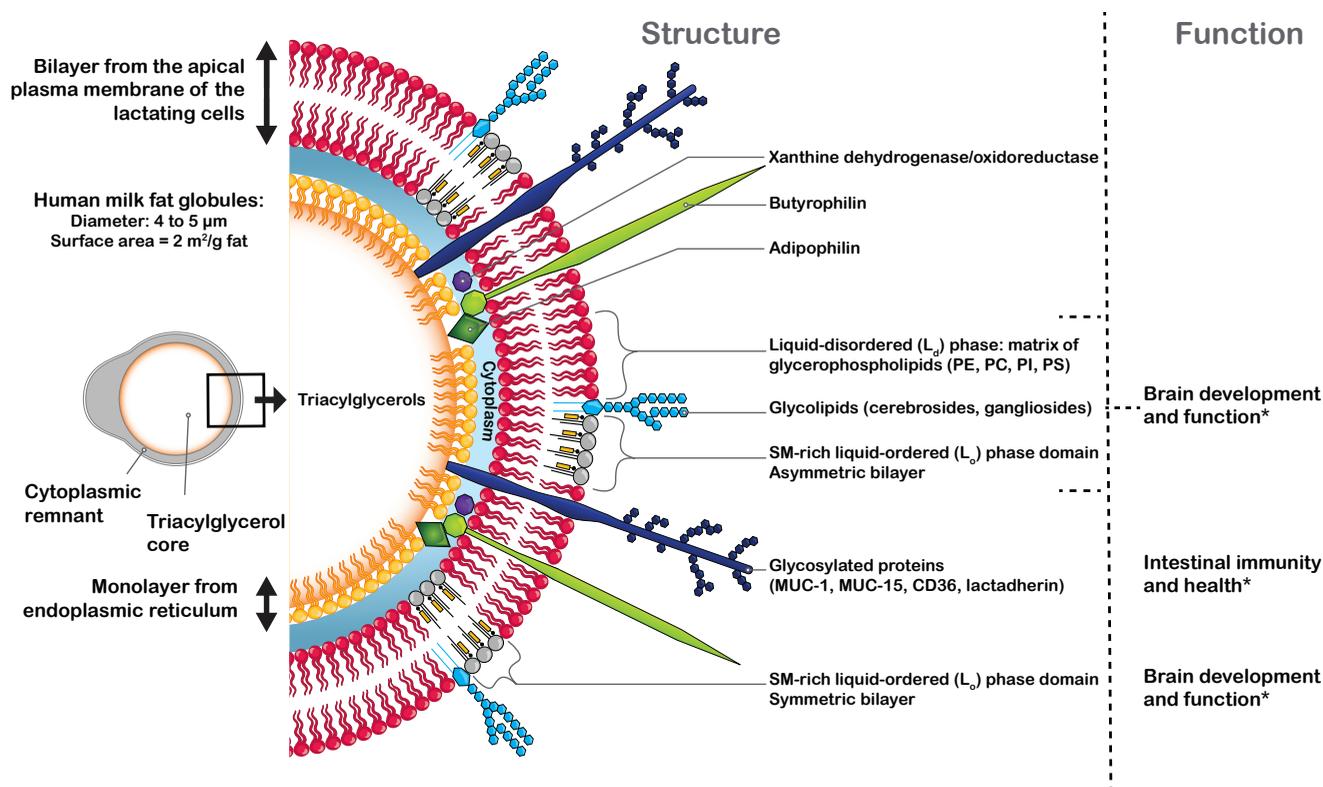
The lipid fraction of MFGM is rich in phospholipids, glycosphingolipids, and cholesterol. Phospholipids make up approximately 30% of the total lipid weight of MFGM, the three most prominent being sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE), which together represent up to 85% of total phospholipids.^{18,27} Phospholipids and sphingolipids play central roles in cerebral neurogenesis and migration during fetal development, as well as promoting neuronal growth, differentiation, and synaptogenesis during the first year of life.^{28,29} Other important polar lipids present in the membrane include the glycerophospholipids phosphatidylserine (PS) and phosphatidylinositol (PI), as well as gangliosides,

which are sphingolipids containing sialic acid and an oligosaccharide side chain. Each of these lipid classes is known to play functional roles within the body, including the support of gut, immune, and central nervous system (CNS) development.³⁰⁻³²

Sphingomyelin

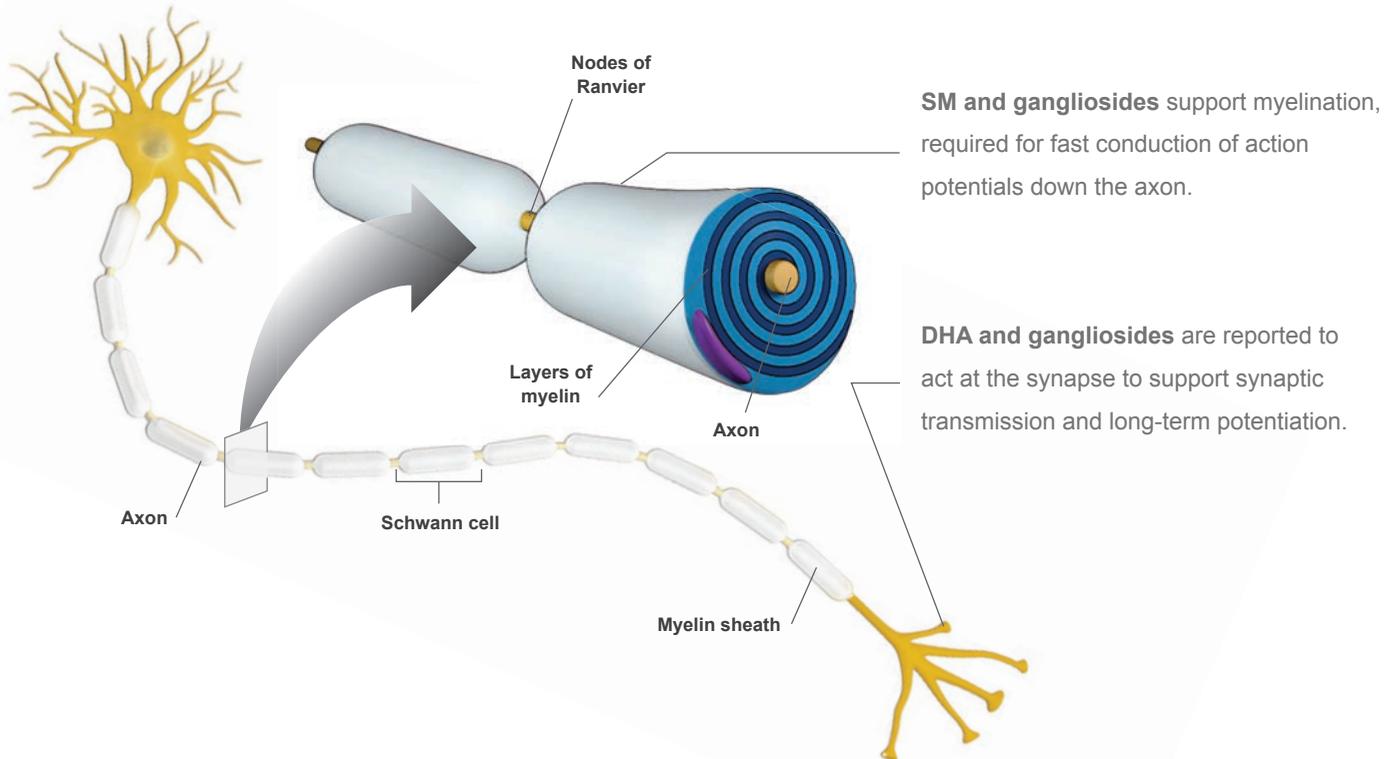
In addition to being a major phospholipid in both human and bovine MFGM, SM is the most abundant species of sphingolipid, and also contains phosphocholine. Its concentration in human milk remains relatively constant throughout lactation; the levels in bovine milk are similar or somewhat lower, while commercially available cow milk-based formulas can contain lower amounts.³³⁻³⁵ SM has been

Figure 2. MFGM: A Trilayer Structure With a Lateral Organization of Polar Lipids^{18,24}



*Scientific understanding of functions of individual MFGM components based primarily on preclinical results

Figure 3. MFGM Components and DHA Support Synaptic Transmission and Myelination^{36-39,51,58,92,93}



shown to play several important roles within the body. In the central nervous system, SM is a key component of the myelin sheath, which insulates axons and supports efficient transmission of nerve impulses.^{36,37} During myelination, nerve axons are wrapped with multiple layers of cell membrane by oligodendrocyte glial cells, a process that accounts for a large portion of brain growth during late gestation and the first two years of life,³⁸ but which can also continue up to 5-10 years of age (Figure 3).³⁹ SM and other sphingolipids are also present in the gut epithelium, where they contribute to membrane structure, modulate growth factor receptors, and provide binding sites for microorganisms, microbial toxins, and viruses.^{40,41} Furthermore, SM and its metabolites (ceramide, sphingosine, ceramide-1-P, and sphingosine-1-P) act as second messengers in cell signaling, with regulatory effects on cell proliferation, cell survival, apoptosis, and inflammation.^{41,42}

Gangliosides

Gangliosides are a complex glycosphingolipid family containing an oligosaccharide side chain plus sialic acid (N-acetylneuraminic acid, NANA). These are quantitatively less abundant in the MFGM relative to phospholipids (9-40 mg/L vs 40-80 mg/L for SM alone).⁴³⁻⁴⁹ Rather than a single compound, gangliosides represent a class of different molecules that vary in the nature and length of their carbohydrate side chains, and the number of attached sialic acids.⁵⁰ The quantity and composition of gangliosides in breast milk varies over the course of lactation, though the GD3 type is most prevalent in human colostrum and mature human milk, as well as in bovine milk.^{46,47} The concentration of gangliosides in bovine milk appears to be similar or somewhat lower than that in human milk.^{44,48}

These lipids are important structural components within the cellular membranes of most body tissues, including nuclear, endoplasmic reticulum, and plasma membranes.⁵¹ They are expressed more predominantly in nervous tissue and are especially abundant in the brain, where they are concentrated within the gray matter and constitute approximately 6% to 10% of the total human brain lipid mass.⁵²⁻⁵⁷ Additionally, gangliosides are enriched at the synaptic membrane of neurons, and are functionally involved in neurotransmission and synapse formation (Figure 3).^{51,58} Brain ganglioside accretion occurs at an accelerated rate in the early years of life, coinciding with the most active period of myelination, axonal outgrowth, and synaptogenesis.^{59,60} Alongside the growth of brain size, total brain ganglioside concentration also increases 3-fold from early fetal development to 5 years of age.⁵⁹

Outside of the central nervous system, ganglioside concentrations are much lower in other organs such as the intestinal mucosa, but may possibly contribute to improved gut microflora and antibacterial defense.⁶¹ Furthermore, gangliosides appear to play a role in cell-cell recognition and adhesion, as well as signal transduction within cell surface microdomains, alongside other sphingolipid components and cholesterol.⁶²

Phosphatidylserine

PS, a glycerophospholipid rich in unsaturated fatty acids, is another major component of MFGM phospholipid. It is present in all cell membranes, and makes up 4% to 15% of total phospholipids in human and bovine milk.^{18,35,63,64} Though susceptible to variation due to analytical methods, PS is generally described to be the fourth most prevalent phospholipid in human milk; its concentration appears to increase as colostrum transitions to mature milk.^{65,66} The levels of PS, as well as total phospholipids, are comparable in human milk versus cow's milk.^{34,35}

Though present throughout the human body, PS is most concentrated in the brain, where it constitutes 15% of the total phospholipid pool; other organs such as lung and kidney

contain lower levels.⁶⁷ It serves a variety of structural and regulatory functions, including contribution to membrane fluidity and therefore the regulation of biological cell activities.⁶⁸ It also supports signal transduction by activating cell surface receptors that react to chemical stimuli, and facilitates membrane-to-membrane fusion, which is a central process in the release of neurotransmitters such as acetylcholine, dopamine, serotonin, and norepinephrine.⁶⁹

Phosphatidylcholine

PC is also present widely in cell membranes, making up approximately 18% to 33% of total phospholipids in human milk^{18,35,63,70} and is one of the two most abundant glycerophospholipids present within MFGM. Bovine milk had similar concentrations of PC to human milk,³³⁻³⁵ and the concentrations of PC as well as free choline in infant formulas were similar to those in bovine milk.³³

PC is also a major structural component of biological membranes, including all plant and animal cells, and is usually the major component of the animal or plant tissue lipid extract known as lecithin. Because PC is a major source of choline, which is also present within milk in free unesterified form, it is involved in the synthesis of SM as well as regeneration of choline and its metabolites, which include the neurotransmitter acetylcholine.⁷¹ In addition to its membrane effects, PC is a key constituent of the intestinal mucus barrier, and has been proposed to exert anti-inflammatory functions in models of colitis as well as arthritis.^{72,73}

Proteins

In addition to the polar lipids, the outer layer of MFGM contains a number of glycosylated and nonglycosylated proteins. Proteomic analysis has revealed at least 191 different known proteins in human MFGM, and comparable numbers in bovine milk protein concentrates (for reference, 133 in buttermilk protein concentrate, 244 in whey protein concentrate).¹⁸ While quantitatively these only represent 1% to 2% of total milk protein content,⁷⁴ MFGM proteins are of significant interest because many are known to

... MFGM proteins are of significant interest because many are known to have bioactive and potentially health-benefiting properties ...

have bioactive and potentially health-benefiting properties; almost half of identified proteins have membrane/protein trafficking or cell signaling functions.⁷⁵ The glycosylated proteins, including mucins (MUC-1, MUC-4, MUC-15), butyrophilin, lactadherin, and CD36, have been suggested to enhance TG digestion efficiency.¹⁸ Furthermore, lactadherin and MUC-1, in addition to the nonglycosylated protein xanthine oxidase, have been shown or suggested in preclinical studies to possess antimicrobial properties.⁷⁵⁻⁸⁰

Carbohydrates

Oligosaccharide concentrations are considerably higher in human milk than in either bovine milk or infant formula.⁸¹ While the great majority of human milk oligosaccharide is in the freely soluble form,^{82,83} within the MFGM structure oligosaccharides are present primarily in the form of conjugates with either glycoproteins or glycolipids, which have been shown to exert a variety of functional activities as outlined above.

Sialic acid represents a class of nine-carbon sugar derivatives of neuraminic acid, the most common of which is NANA. Sialic acid is widely distributed in animal tissues, sometimes in free form but primarily as a component of gangliosides (bound to the oligosaccharide side chain) and glycoproteins; the

highest known concentration occurs in the human brain.⁸⁴ In human milk, sialic acid is primarily bound to oligosaccharide rather than glycoprotein (ratio 3:1), whereas in typical infant formula total sialic acid levels are <25% of that in breast milk, and the majority is protein-bound (ratio 1:3).⁸⁵ The MFGM glycoproteins MUC-1 and MUC-15 are high in sialic acid content.⁸¹

Breast-fed infants have significantly higher amounts of sialic acid in saliva,⁸⁶ and 22% higher concentrations of protein-bound sialic acid in brain frontal cortex, compared with formula-fed infants.⁸⁴ Evidence has suggested a contributory role for sialic acid in synaptic transmission, as well as a number of other functions during brain development.^{31,57,87}

Scientific Evidence for Health Benefits of MFGM

A. Advances in MFGM Research and Technology

While much is still being learned about the significance of MFGM and its components to infant nutrition, this research rests on over two decades of foundational research into milk lipid composition and function.⁸⁸ Emerging analytical methods in the 1970s allowed researchers to characterize the specific content of fatty acids within the MFGM of both human and cow milk, leading to the recognition of SM and other phospholipids as major components of the membrane.^{89,90} During the 1980s, further studies compared the changes in milk phospholipid composition across the full lactation cycle,⁷⁰ and further characterized the relative compositions of SM, other phospholipids, and gangliosides in human milk, bovine milk, and infant formula. This research contributed to the recognition that these components may play important roles in growth, brain, and immune development during the critical period of early infancy.^{33,44,64,70} At the same time, advances in dairy processing technology have facilitated the process of purifying and concentrating MFGM from bovine milk, allowing for ingredients enriched in milk polar lipid and other MFGM components to be added to infant formulas.^{24,88} Thus, there has been parallel

... advances in dairy processing technology have facilitated the process of purifying and concentrating MFGM from bovine milk ...

development in both the scientific interest surrounding bioactivity of MFGM components, and the technological advances that make preclinical and clinical research studies possible.

B. MFGM and Components: Preclinical Trial Data

1. Benefits to Brain Development and Cognitive Function

The Developing Brain

Metabolic activity is highest in infancy and early childhood, reflecting the energy demands associated with rapid growth and development during this time period, though the brain still remains highly metabolically active with remodeling that continues well into adult life.⁹¹ Important insights can thus be derived from basic research into the neural effects of bioactive nutrients not only during early development, but at different stages throughout life. A number of preclinical studies using various animal and *in vitro* models have elucidated some of the roles for MFGM and MFGM-derived components in the development of brain structure, learning, memory, and other cognitive functions.

Sphingomyelin

Loss of myelination results in defects of nerve impulse conduction, as seen in human multiple sclerosis as well as animal models of neural disease.³⁶ In developing rats with impaired SM synthesis, dietary SM was shown to restore myelination of the central nervous system, as measured by brain weight, myelin weight and thickness, and axon diameter (Figure 3).³⁷

Gangliosides

Double knockout mice who lack brain gangliosides have shown severe disruptions in axon myelination, demonstrating that gangliosides are critical to axon stability and function (Figure 3).^{92,93} Other mice deficient in specific gangliosides demonstrated impaired stability of paranodal junctions in myelinated nerve fibers and a slowing of nerve conduction.⁹⁴ Furthermore, *in vitro* and *in vivo* studies in different animal models suggest that parenteral administration of ganglioside (by subcutaneous, intraperitoneal, and intraventricular injections) supports learning and memory.⁹⁵⁻⁹⁹ In other studies, ganglioside administration to rats with aging or compromised brain function appeared to alleviate genetic and lesion-induced memory deficits and improve spatial learning and memory,^{100,101} though lack of benefit has also been seen in this model.¹⁰²

Phosphatidylserine

There is some evidence from adult animal models suggesting that PS may attenuate some of the neural effects of aging. In rats, long-term PS supplementation (oral or intraperitoneal) was found to diminish some of the neural changes usually seen in aging rodents, such as loss of dendritic spines and decrease in neurotransmitter release.^{103,104} Similarly, chronic oral PS supplementation also improved spatial memory and passive avoidance retention in old rats with age-associated cognitive dysfunction.¹⁰⁵

Phosphatidylcholine and Choline

Studies in pregnant rodents have shown that PC supplementation supports learning and memory in offspring.¹⁰⁶ As noted, hydrolysis of membrane PC can also generate free choline, though the rates of such conversion are not easily defined.⁷¹ In a mouse model, experimental inhibition of choline uptake and metabolism in embryos was associated with neural tube defects.¹⁰⁷ In rats, supplementation with choline improved memory and learning,¹⁰⁸ and the most positive effects of oral choline on brain function occur during the periods of peak neurogenesis

and synaptogenesis, which when extrapolated to humans would correspond to a period beginning *in utero* and continuing to 4 years of age.¹⁰⁹

Sialic Acid

An *in vivo* model demonstrated that adding sialic acid to the diet of piglets improves learning and memory.¹¹⁰ This finding also coincided with a dose-related increase in the amount of sialylated glycoproteins in the frontal cortex. In another model, malnourished rat pups who were injected intraperitoneally for 7 days with sialic acid were found to have increased brain ganglioside and glycoprotein sialic acid concentrations and a decrease in behavioral abnormalities.⁹⁶ When later tested in a Y maze as adults, the sialic acid-treated rats learned the maze more quickly than control rats.

Combination of MFGM Components

A number of preclinical studies have also been conducted using MFGM and combinations of MFGM-derived components. In a recent study by Liu et al (2014), neonatal piglets were fed formulas containing 0% (control), 0.8%, or 2.5% of PL-20, a supplement providing milk phospholipids and gangliosides, from postnatal day 2 to 28.¹¹¹ According to the authors, the 0.8% concentration was used to mimic breast milk, whereas the 2.5% concentration was used to assess the effects of providing phospholipids and gangliosides at amounts well beyond the human milk range. Piglets that were fed 0.8% and 2.5% PL-20 made choices more rapidly and with fewer errors in a spatial T-maze cognitive test compared to controls, implying improved spatial learning. Mean brain weight in the piglets fed PL-20 was also 5% higher, with multiple brain areas having more gray and white matter than control piglets, suggesting that the additional phospholipids and gangliosides were incorporated into the developing brain, promoting myelination and growth. Similarly, Vickers et al (2009) demonstrated that administration of complex milk lipids to rats from postnatal day 10 through adulthood (day 80) led to significant improvements in learning and memory

tasks compared to control animals.¹¹² Conversely, a study of complex milk lipid supplementation to pregnant mice did not have an effect on cognitive tasks in their offspring.¹¹³

2. Benefits to Immunity and Gut Health

The Developing Immune System and Gut

Like the central nervous system, other organ systems also exhibit rapid development and functional adaptation during infancy and early childhood. In particular, the gastrointestinal tract is essential in relation to both overall growth and the development of immunity and tolerance, as it provides both the absorption site for all nutrients as well as a major interface for immune interaction with the environment.⁶ A number of preclinical studies of MFGM and its components have illustrated beneficial roles in the maintenance of gut health and immunity, via mechanisms that include immune modulation as well as direct antipathogenic activity.

Sphingomyelin

Sphingolipids, including SM, are present in the apical membrane of the gut epithelium, and are involved in cell regulation.⁴⁰ They are also important for maintaining membrane structure, modulating growth factor receptors, and serving as binding sites or competitive binding inhibitors for microorganisms, microbial toxins, and viruses.⁴¹ In one study, oral SM was associated with more rapid intestinal maturation in rats.¹¹⁴ In another rodent model, SM was associated with beneficial effects on colon tumorigenesis.¹¹⁵ Evidence for the effects of SM on inflammation is conflicting, as dietary supplementation has been found to both increase¹¹⁶ and decrease¹¹⁷ inflammation in different mouse models of colitis.

Gangliosides

Gangliosides are also present in intestinal mucosal cell membranes, though at lower concentrations than within the CNS.⁵⁷ Gangliosides have been shown *in vitro* and in rabbit intestinal loops to prevent the binding of enterotoxins to intestinal cells, which may contribute to protection against

bacterial disease.⁴⁴ Other preclinical studies have shown that gangliosides contribute to improved gut microflora, gut immunity, and defense against infections.⁶¹ Dietary supplementation with GD3, a ganglioside species enriched in human colostrum as well as bovine MFGM, decreased the incidence and pathologic severity of necrotizing enterocolitis (NEC) in newborn rats, in part by modulating the mucosal immune response in favor of anti-inflammatory cytokines such as IL-10.¹¹⁸

Sialic Acid

In vitro studies suggest that sialic acid, in addition to being a structural component of gangliosides, may also influence the intestinal immune response by acting as a decoy receptor or competing with pathogens for receptor sites on intestinal epithelial cells.¹¹⁹

Phosphatidylcholine

Though also a cell membrane component, PC represents more than 90% of the phospholipid in the intestinal mucus layer, and therefore may contribute to intestinal defense against invasive pathogens. *In vitro*, PC protected against *C. difficile* toxin-induced intestinal barrier injury,¹²⁰ and was shown to attenuate neutrophil activation.¹²¹

Protein Components of MFGM

There are several bioactive protein components of the MFGM, including the glycoproteins lactadherin, MUC-1, and butyrophilin, which have been shown in preclinical studies to affect immune response.¹²² These components influence the immune system by several mechanisms, including interference with microbe adhesion to intestinal epithelia, bacteriocidal action, support of beneficial microbiota, and modulation of other parts of the immune system.³⁰ Mice that were fed prophylactically with bovine whey glycoprotein fraction, including MFGM proteins, did not develop diarrhea after exposure to rotavirus.¹²³ Lactadherin is a cell adhesion molecule that interacts with integrins and is associated with the membrane through binding to PS. Depletion of lactadherin led to intestinal mucosal injury in mice, while

in vitro administration of lactadherin promoted experimental wound healing of intestinal epithelial cells.¹²⁴

MUC-1 is a rod-like glycoprotein on the surface of MFGM and many epithelial cells that can act as a decoy for binding of infective agents. Bovine MUC-1 was found to inhibit rotavirus infectivity in human cell lines, including Caco-2 cells.^{76,77} It also prevented the appearance of antiviral antibodies in the serum of mice after oral challenge with rotavirus.⁷⁶ MUC-1 has also been shown to inhibit binding of S-fimbriated *E. coli* to buccal epithelial cells, and both MUC-1 and lactadherin are resistant to digestion in the infant stomach, a property which is likely relevant to their functional effects in supporting gut immunity.¹²⁵

MFGM-derived xanthine oxidase was demonstrated to exert direct antibacterial effects against several pathogens *in vitro* due to the generation of hydrogen peroxide under specific conditions.⁷⁹ While there is no known evidence for direct antimicrobial activity of butyrophilins, some data suggests that proteins of this family may be costimulatory molecules which contribute to immune homeostasis.¹²⁶

Combination of MFGM Components

In light of the functions demonstrated by its constituents, MFGM may be capable of modulating immune function in the gut through distinct but potentially complementary mechanisms. Glycosylated proteins (MUC-1, MUC-15, butyrophilin, and lactadherin) and glycosylated sphingolipids from MFGM may promote the development of healthy infant gut microbiota by favoring beneficial Bifidobacterium species.¹²⁷ Another key to the immunomodulatory function of MFGM may be that its structure is similar to that of the intestinal cell membrane, allowing human milk glycans (including those on glycoproteins and glycolipids) to competitively inhibit the binding of pathogens (bacteria, viruses, even toxins) to host cells.¹²⁸

A number of preclinical studies have demonstrated inhibitory effects of MFGM against several pathogens. Both whole

bovine MFGM and its extracted lipid components were found to exhibit dose-dependent inhibition of rotavirus infectivity *in vitro*.¹²⁹ Antibacterial effects of MFGM have included decreased gastric colonization and inflammation after *H. pylori* infection in mice;⁸⁵ inhibition of shiga toxin gene expression by *E. coli* O157:H7;¹³⁰ and decreased colonization and translocation of *L. monocytogenes*.¹³¹

In summary, a significant body of preclinical data have demonstrated that MFGM, or components thereof, play roles in neural development and function, gastrointestinal immune defense, and gut health.

C. MFGM and Components: Clinical Trial Data

The safety and efficacy of formula supplemented with MFGM, complex milk lipids, or MFGM components in infants and young children has been investigated in several randomized, controlled trials. In line with preclinical findings, most of these studies have demonstrated evidence for clinically relevant benefits in terms of cognition, behavior, and gut health and immunity.

1. Safety and Tolerance

As a component naturally present in human and bovine milk, MFGM has a long history of safe use in infants and young children; exogenous supplementation of MFGM has also demonstrated safety and tolerability in infant and adult trials.^{25,132-135} A multicenter, randomized, controlled trial investigated the safety and tolerance of MFGM-enriched formulas to 12 months of age,¹³³ comparing standard formula to two investigational formulas, one enriched with a lipid-rich and the other with a protein-rich bovine MFGM fraction. Overall, all three groups demonstrated normal growth, with

... preclinical data have demonstrated that MFGM, or components thereof, play roles in neural development and function, gastrointestinal immune defense, and gut health ...

no significant differences in formula tolerance.²⁶ Adverse events were similar across groups except for a higher rate of eczema in the protein-rich MFGM group. However, in another trial infants fed an experimental MFGM-containing formula until 6 months of age also demonstrated normal growth at 12 months (Table 1),¹³⁶ and reported no safety or tolerance concerns, including no significant differences in incidence or prevalence of rash compared to either control formula or a breast-fed reference group.^{25,137} Clinical studies have also demonstrated the safety and tolerance in infants of formulas containing MFGM-derived complex milk lipids¹³⁸ or SM.¹³⁹ Similarly, safety and tolerance of MFGM-supplemented complementary foods have been demonstrated in infants and young children through 6 years old,^{134,135} and adults.¹⁴⁰

2. Benefits to Brain, Cognitive, and Behavioral Development

Several studies of diets supplemented with MFGM and its components, including gangliosides and SM, have aimed to address measures of cognitive development in pediatric populations.^{135,136,138,139} These trials have been conducted in both infants and young children of different age groups and are summarized in Table 1.

Individual MFGM Components

Sialic acid has been suggested as a potential factor in breast milk supporting optimal brain development and function, though there is limited human data.⁵⁷ Breast-fed infants have significantly higher levels of salivary sialic acid than formula-fed infants.¹⁴¹ While both breast-fed and formula-fed infants have significant amounts of ganglioside in brain tissue, protein-bound sialic acid was found to be 22% higher in the frontal cortex gray matter of breast-fed infants compared to formula-fed infants,⁸⁴ echoing similar findings in an animal study.¹¹⁰ No clinical trial data is available to date.

PS has been clinically evaluated for neurocognitive benefits, though no trial has targeted infants or young children. In a small randomized, controlled trial (N=36) of children ages 4 to 14 with attention deficit hyperactivity disorder (ADHD), dietary supplementation with PS (200 mg per day) for 2 months led to improvement of symptoms of ADHD as well as short-term auditory memory.¹⁴² In an adult study of cortical activity after mental stress, dietary supplementation with PS (for 6 weeks) led to a more relaxed state compared to subjects on a control diet.¹⁴³

Combination of MFGM Components

*Tanaka et al, 2013*¹³⁹

The neurobehavioral effects of feeding formula supplemented with SM-enriched phospholipid were assessed in a trial of preterm infants.¹³⁹ In this double-blind controlled trial, very low birth weight preterm infants (birth weight <1500 g) were randomized either to a control group (n=12) who were fed formula containing phospholipids derived from egg yolk lecithin with SM at 13% of total phospholipid or to a test group (n=12) fed formula with milk-derived phospholipids containing 20% SM. The total amount of phospholipid added was the same for both groups, and the SM contents were both lower than that reported for mature preterm milk (42.4%±8%).¹⁴⁴ Infants in the trial group had significantly higher percentages of SM in total phospholipids after 4, 6, and 8 weeks of feeding compared to those in the control group.

Table 1: Clinical Studies Reporting Cognitive or Behavioral Benefits of MFGM and MFGM Components

CLINICAL STUDY	POPULATION	MAIN FINDINGS
Timby et al (2014) ¹³⁶	Full-term infants	Feeding infants a formula supplemented with MFGM until 6 months of age improved their cognitive performance at 12 months relative to control infants, and resulted in similar performance to breast-fed infants.
Gurnida et al (2012) ¹³⁸	Healthy term infants	Feeding infants a formula supplemented with MFGM-derived complex milk lipids until the age of 6 months resulted in motor skills and cognitive performance similar to breast-fed infants, and better than infants fed the unsupplemented formula. Increased serum ganglioside levels in the supplemented group, not different than the breast-fed group.
Tanaka et al (2013) ¹³⁹	Very low birth weight preterm infants	Very low birth weight preterm infants showed improvements across multiple developmental measures, when fed with formulas supplemented with phospholipids enriched for SM over a period of 8 weeks compared to formulas with lower SM content. Increased red blood cell SM levels in the supplemented group.
Veereman-Wauters et al (2012) ¹³⁵	Healthy preschool children	Preschool children who consumed MFGM-enriched chocolate milk formula for 4 months were rated to have better behavioral and emotional regulation by their parents compared to those fed a standard formula.

Mental and psychomotor development was assessed using the Bayley Scales of Infant Development II (BSID-II), including the Behavior Rating Scale (BRS) at 6, 12, and 18 months of corrected age. Visual recognition memory was assessed with the Fagan test score (novelty preference rate). While the two groups did not differ in Bayley Mental Development Index (MDI) and Psychomotor Development Index (PDI) scores, the test group had significantly higher BRS scores than the control group at 12 and 18 months, including orientation, emotional, motor quality, and overall scores. Additional significant differences in outcomes included higher scores on the Fagan test at 12 months and the Sustained Attention Test at 18 months in the high-SM group compared to the control

group.¹³⁹ This pilot study suggested a correlation between dietary SM content and neurobehavioral development in very low birth weight preterm infants.

Gurnida et al, 2012¹³⁸

Another study addressed the cognitive effects of formula supplemented with a ganglioside-enhanced, MFGM-derived complex milk lipid in term infants.¹³⁸ In this randomized, double-blind, controlled trial, healthy infants (2-8 weeks of age) were assigned to receive either standard infant formula (control, n=30) containing 6 mg gangliosides per 100 g, or a supplemented infant formula (test, n=29) with added complex milk lipids to increase ganglioside concentration to 9 mg per 100 g, until 6 months of age. The level of gangliosides (measured as GD3 only) in the supplemented infant formula was approximately 11-12 µg/mL. This level is within the range of total ganglioside levels in human milk (3.4-16.2 µg/mL).^{44,45,47} A breast-fed reference group (n=32) was also included. Results showed that serum ganglioside levels in the test group (measured as GM3, GD3, and total ganglioside) were significantly higher than controls at 6 months, but did not significantly differ from levels in the breast-fed group.

Cognitive outcomes were measured at 6 months of age using the Griffiths Mental Development Scale, a validated screening tool which provides assessment across five domains, including Locomotor, Personal-Social, Hearing and Speech, Eye and Hand Coordination, and Performance.¹⁴⁵ The test group had significantly increased scores for Hand and Eye Coordination, Performance, and Total Score (General IQ) at 6 months compared to the control group, but there were no significant differences in cognitive performance compared to the breast-fed reference group.¹³⁸ The authors concluded that “ganglioside supplementation to more closely match the intake of breast-fed infants may provide some advantages in cognitive development, particularly those aspects related to motor skills.” However, the positive effects cannot be solely attributed to gangliosides since the supplement used in this trial contained other polar lipids as well.

Timby et al, 2014¹³⁶

A recent clinical study was conducted to determine potential impact of supplementation with MFGM on cognitive

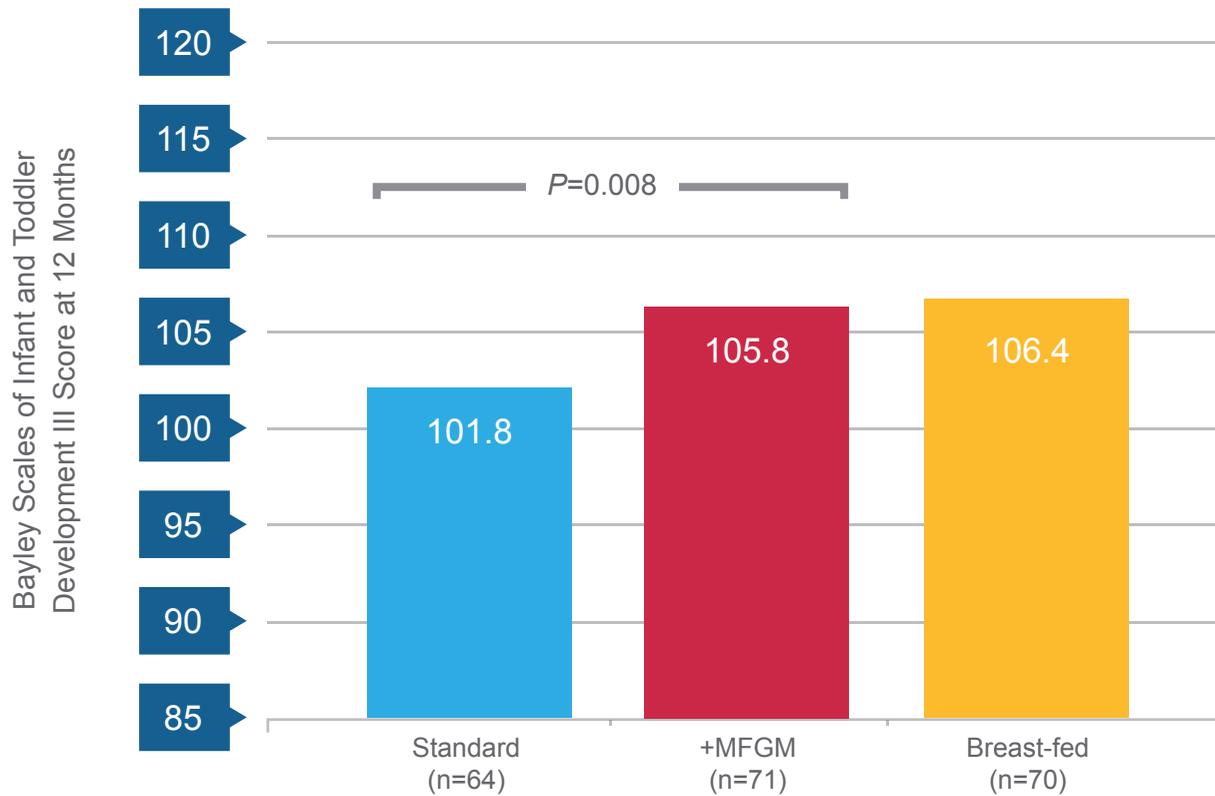
development in infants.¹³⁶ In this randomized, double-blind trial, term infants (<2 months old) were assigned to consume either a standard formula (n=64) or an MFGM-supplemented test formula (n=71) until 6 months of age. Formula was supplemented with MFGM (in the form of MFGM-10, Arla Foods Ingredients, Denmark), providing 4% of total protein content as MFGM protein. A breast-fed reference group (n=70) was also included. Cognitive assessment was done using the Bayley Scales of Infants and Toddler Development-III (BSID-III) at 12 months of age. The MFGM-fed infants exhibited mean cognitive scores that were significantly higher compared to the control group (105.8 vs 101.8; $P<0.008$) (Figure 4), and not significantly different from the breast-fed reference group. In contrast, there were no significant differences in motor domain scores between the three groups, and both experimental and control formula groups scored lower than the reference group in the verbal domain. The authors concluded that supplementation of infant formula with MFGM demonstrated a positive effect on cognitive function, and “eradicated the gap in cognitive performance between breast-fed and formula-fed infants at 12 months of age,” noting also that the cognitive effect was persistent and measurable 6 months after the end of formula feeding. Further, the four-point difference in Bayley cognitive scores is clinically relevant, being similar in size to the increment previously described in the meta-analysis of Anderson et al for better cognitive performance in breast-fed compared to formula-fed infants.¹⁴⁶

Veereman-Wauters et al, 2012¹³⁵

The potential behavioral benefits of MFGM supplementation have also been examined in young children. In another randomized, double-blind, controlled trial, healthy preschool children (2.5 to 6 years of age) consumed a fortified milk beverage for a period of four months. The beverage for the control group (n=97) contained 60 mg/day of endogenous phospholipid, while the test group beverage (n=85) contained a total of 500 mg/day of dairy-derived phospholipid due to the addition of MFGM concentrate (provided as INPULSE, Bullinger SA, Belgium).¹³⁵ At the end of the trial, a validated behavioral questionnaire, the Achenbach System of

Figure 4: MFGM Supplementation Supports Cognitive Function in Infants¹³⁶

Healthy term infants were fed formula with or without added MFGM to 6 months of age, then evaluated (Bayley III) at 12 months of age



Supplementation of infant formula with MFGM improved measures of cognitive development compared to standard formula.

Empirically Based Assessment (ASEBA) was completed by parents and teachers. The ASEBA questionnaire is considered a gold standard for assessing emotion and behavior in preschool children;¹⁴⁷ it encompasses internal problem (emotional, anxious/depressed, somatic, and withdrawn), external problem (attention and aggressiveness), and total problem scores that are standardized for gender and age. The investigators observed significant differences in internal, external, and total behavioral problem scores in favor of the test formula group, as reported by parents (but not by teachers).¹³⁵

In summary, current clinical evidence suggests that dietary supplementation of MFGM or its components may play a beneficial role in supporting healthy cognitive development, cognitive function, and behavioral regulation in infants and young children.

3. Benefits to Immunity and Gut Health

A difference in the incidence of infectious outcomes, particularly gastroenteritis and acute otitis media, has been consistently reported between formula-fed and breast-fed infants.^{148,149} As with preclinical studies, clinical trials have also demonstrated that MFGM and several of its components are associated with beneficial effects on promoting gut health and reducing infection. Possible mechanisms of action include modulation of the immune system, altering the composition or function of the gastrointestinal tract microbiota, or a combination thereof, reflecting the complex interplay between the gut microbiota and the immune system.¹⁵⁰

Since the levels of various MFGM components found in infant formula, such as SM, sialic acid, and gangliosides, may vary considerably from the average amounts found in breast milk,^{33,47,141} it is possible that supplementation of formula with MFGM and its derived components may result in a composition of infant formula closer in some respects to that of human milk, which in turn may potentially support outcomes to cognition, immunity, and gut health closer to those reported in breast-fed infants.

Gangliosides

The effect of ganglioside-supplemented formula on gut microbiota was investigated in a randomized trial of preterm infants. Infants who were fed formula supplemented with gangliosides at a concentration of 1.43 mg per 100 kcal had significantly reduced mean counts of pathogenic *E. coli* at 3 and 7 days after birth, and a lower percent of fecal samples positive for *E. coli* at 7 and 30 days, compared to those fed control formula. Infants in the supplemented group also had increased mean bifidobacteria counts at the end of the 30-day trial compared with controls (Table 2).¹⁵¹ These findings suggest a possible role for gangliosides in supporting the development of beneficial gut flora. However, it should be noted that the ganglioside preparations used in this trial

were porcine-derived and may not necessarily reflect the ganglioside profile present in MFGM.¹⁵²

Lactadherin

A cohort study evaluated the correlation between breast milk levels of the MFGM protein lactadherin and symptomatic rotavirus infection.¹⁵³ Increased concentrations of lactadherin in the breast milk of 200 mothers in Mexico City were associated with significantly reduced symptoms of rotavirus infection in their breast-fed infants. This finding remained significant after adjusting for breast milk levels of secretory IgA, which is known to have antirotaviral activity (Table 2).¹⁵³ However, the dietary effect of lactadherin supplementation has not been independently evaluated in any clinical trial.

Combination of MFGM Components

The previously described study by Timby et al, demonstrating improved measures of cognition in term infants receiving MFGM supplementation, also was analyzed for disease symptoms and medication use in the first year of life.²⁵ In particular, the cumulative incidence of acute otitis media was analyzed between the two randomized feeding groups (standard formula or formula supplemented with MFGM-10 to 6 months of age), and compared to a breast-fed reference group. The MFGM-supplemented group experienced a significant reduction in episodes of acute otitis media up to 6 months of age compared with infants fed standard formula (1% vs 9%; $P=0.034$); with no difference in otitis media incidence compared to the breast-fed group (0%). In addition, a significantly lower incidence and longitudinal prevalence of antipyretic drug use was seen in the MFGM-supplemented group (25%) compared with the standard formula group (43%) (Table 2, Figure 5). No differences were seen for other antibiotic-treated bacterial infections. Differences in serum antipneumococcal IgG concentrations were also found, leading the authors to speculate that supplementation with MFGM may reduce the risk of otitis media through modulatory effects on the humoral immune system.

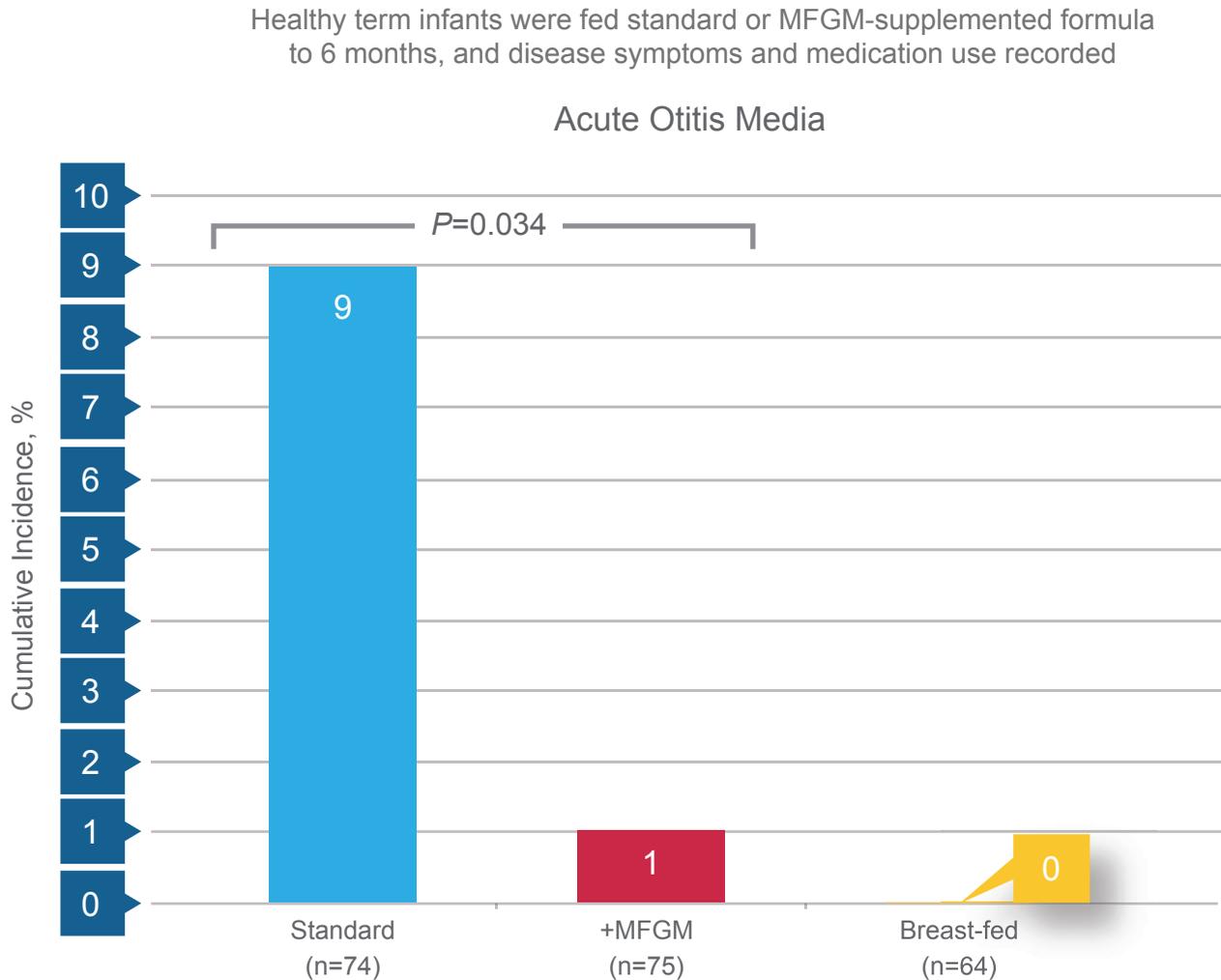
Table 2: Clinical Studies Reporting Benefits of MFGM and MFGM Components on Gut Health and Other Health Outcomes

CLINICAL STUDY	POPULATION	MAIN FINDINGS
Zavaleta et al (2011) ¹³⁴	Healthy infants and young children	Daily intake of complementary food containing MFGM as the protein source, for 6 months, reduced the prevalence of diarrhea and bloody diarrhea compared to the food containing skim milk protein.
Timby et al (2015) ²⁵	Full-term infants	Feeding infants a formula supplemented with MFGM until 6 months of age reduced the incidence of acute otitis media, from inclusion until 6 months of age, relative to control infants, but resulted in similar incidence to breast-fed infants. It also decreased the incidence and longitudinal prevalence of antipyretic use compared to control infants during the intervention period.
Veereman-Wauters et al (2012) ¹³⁵	Healthy preschool children	Young children fed a chocolate milk formula supplemented with MFGM, for a period of 4 months, had reduced febrile episodes compared to those fed the standard formula.
Newburg et al (1998) ¹⁵³	Breast milk from mothers and rotavirus infection in breast-fed infants	Lactadherin concentrations in breast milk showed a significant inverse association with symptoms of rotavirus-infected breast-fed infants, independently of the concentrations of the secretory IgA in human milk.
Rueda et al (1998) ^{151,152}	Healthy preterm infants	A formula supplemented with gangliosides resulted in reduced fecal <i>E. coli</i> counts and increased fecal counts of bifidobacteria throughout 30 days of postnatal age.

Another clinical trial evaluated the effects of an MFGM-enriched complementary food on health outcomes in infants older than 6 months.¹³⁴ This randomized, double-blind controlled trial enrolled 499 primarily breast-fed term infants in Peru at 6 to 11 months of age. Infants were assigned to receive a daily milk-based complementary food that included either whey protein concentrate enriched in MFGM (provided as MFGM-10, Arla Foods Ingredients, with an average daily intake of 5.9 g), or an equal amount of additional protein from skim milk (control) for 6 months. Results showed that the group with the MFGM-supplemented diet had a significantly

lower prevalence of diarrhea during the study compared to the control group (means, 3.84% vs 4.37%; $P < 0.05$), as well as a significant reduction (46%) in episodes of bloody diarrhea compared to controls ($P = 0.025$). The most common pathogen isolated during the diarrhea episodes was *E. coli* (45% of pathogens) (Table 2).¹³⁴ The authors concluded that the addition of an MFGM-enriched protein fraction to complementary food could have beneficial effects on severe diarrhea in infants, especially in vulnerable populations.

Figure 5. MFGM and Otitis Media Risk in Infants²⁵



Formula supplementation with MFGM reduced the risk of acute otitis media and was associated with decreased antipyretic use during the intervention

In addition, the previously described study by Veereman-Wauters et al in preschool-age children (2.5 to 6 years old) also reported the effect of MFGM-enriched formula consumption on health outcomes. In this randomized controlled trial, the group of children receiving the MFGM-enriched milk beverage reported a significant reduction in the number of days with fever, and particularly the number of short febrile episodes (<3 days), compared to the control group (Table 2).¹³⁵

Summary and Conclusions

Optimal nutrition during the early years of life is essential to healthy growth and development. Past and ongoing research into human milk has expanded our understanding of its composition, function, and variability, as well as the nutritional needs of infants. A considerable number of studies have identified MFGM as a complex and unique structure present in human and bovine milk, and a source of multiple bioactive compounds, including phospholipids, glycolipids, glycoproteins, and carbohydrates that have important functional roles within the brain, gut, and elsewhere in the body. An increasing body of evidence supports both safety and efficacy of MFGM and its constituents. Preclinical studies have demonstrated effects of MFGM-derived bioactive components on brain structure and function, intestinal development, and immune defense. Similarly, pediatric clinical trials have reported beneficial effects on cognitive and immune outcomes. In populations ranging from premature infants to preschool-age children, dietary MFGM supplementation has been associated with clinically relevant improvements in cognition and behavior, gut bacterial flora

composition, fever incidence, and infectious outcomes including diarrhea and otitis media. In conclusion, available evidence shows that dietary supplementation of MFGM or its key components can play a role in supporting nutrition and health in infants and young children. In particular, for infants the addition of MFGM to formula may help narrow the nutritional and functional gap between infant formula and human milk.

... addition of MFGM
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Appendix: Infant or Child Cognitive Developmental Assessments

TEST	DESCRIPTION
The Bayley Scales of Infant Development, currently in the 3rd edition (BSID-III) ¹⁵⁴	Evaluates mental and psychomotor development of infants, and is a standardized test tool that is widely used in clinical research.
The Fagan Test of Infant Intelligence ¹⁵⁵	Determines novelty preference rate. A higher novelty preference rate indicates better intellectual development. There is some evidence that intellectual development can be predicted from the speed of habituation and the novelty preference rate.
Visual Evoked Potentials (VEPs)	Measures the neurotransmission speed via the optic nerves. Latency (ms) of 16-Hz pattern stimulation is measured to evaluate the level of myelination.
Sustained Attention Test ¹⁵⁶	Measures attention during 4 minutes of free play.
Griffiths Mental Development Scale ¹⁴⁵	Assesses multiple aspects of cognitive development. There are five scales: locomotor, personal-social, hearing and speech, eye and hand coordination, and performance. A numeric score is produced from these, and a General IQ (total) is produced from the five scores.
The Achenbach System of Empirically Based Assessment (ASEBA) ¹⁵⁷	Assesses competencies, strengths, adaptive functioning, and behavioral, emotional, and social problems.
Wechsler Intelligence Scale for Children, currently in the 5th edition ¹⁵⁸	Generates a Full Scale IQ, which represents a child's general intellectual ability. It also provides five primary index scores (ie, Verbal Comprehension Index, Visual Spatial Index, Fluid Reasoning Index, Working Memory Index, and Processing Speed Index) that represent a child's abilities in more discrete cognitive domains.

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