Functional glycans and glycoconjugates in human milk$^{1-3}$

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ABSTRACT

Human milk contains complex carbohydrates that are important dietary factors with multiple functions during early life. Several aspects of these glycostructures are human specific; some aspects vary between lactating women, and some change during the course of lactation. This review outlines how variability of complex glycostructures present in human milk is linked to changing infants’ needs. Am J Clin Nutr 2013;98(suppl):578S–85S.

INTRODUCTION

Milk from a mother’s mammary glands is the natural exclusive food for all newborn mammals, including humans. Human milk is tailored by natural selection to suit the needs of human infants, which differ in many aspects, for example, from the needs of newborn calves. Not surprisingly, therefore, human milk is different from cow milk. Cow-milk–based infant formula for human use is adapted to mimic human-milk composition and to fulfill human infants’ needs for healthy growth. However, there are numerous characteristics of human milk that are difficult to incorporate in formulas that are based on cow milk or on other animal and plant raw material. One of these is the human-specific and variable glycan composition. In addition to lactose, the common milk sugar, glycans that are found in milk include structurally more complex oligosaccharides in free form or in covalently bound form as glycolipids, glycoproteins, glycopeptides, and glycansaminoglycans. From recent research these complex glycostructures emerge as important dietary factors during early life with multiple functions. This review discusses the biological relevance of structural and temporal variability in the following classes of human-milk glycans: 1) free oligosaccharides, 2) glycolipids, and 3) glycoproteins.

FREE OLIGOSACCHARIDES IN HUMAN MILK

Of all of the milk glycans, only the disaccharide lactose is a direct source of energy for the human newborn. It is made accessible by endogenous human enzymes breaking down and converting lactose to glucose in epithelial cells of the small intestine.

General human-milk oligosaccharide structure and function

In contrast to lactose, from which they are derived, human-milk oligosaccharides (HMOSs)$^4$, consisting of 3 to $>50$ monosaccharide units, are thought to be indigestible by endogenous enzymes in human infants and to remain mostly intact until they reach the large intestine (1, 2). HMOSs in unbound form are the fourth most abundant substance class in human milk after water, lactose, and fat. During their passage through the oral cavity, pharynx, stomach, small intestine, and finally the colon, they prohibit the attachment of pathogenic microorganisms to human epithelia by acting as preferred binding sites for pathogenic microorganisms that would otherwise be able to bind to oligosaccharide units of host cell surface glycoproteins and glycolipids and establish an infection (3, 4).

Sialic acids comprise a family of 9-carbon sugars that occur in all mammalian milks studied so far. HMOSs are a rich source of the sialic acid N-acetylneuraminic acid (Neu5Ac). Sialic acids also comprise the terminal functional residue of brain gangliosides and glycoproteins, such as the neural cell adhesion molecule, and have important roles in the development of the infant brain (5). There is evidence from animal models that dietary sialic acids are readily incorporated into the developing brain and enhance learning ability (6, 7). In piglets, a diet rich in the sialic acid containing sialyllactose increased the amount of brain sialic acid as well as the expression of 2 learning-related genes and enhanced learning and memory (7). Therefore, sialic acids from ingested milk may be absorbed as such in the small intestine or catabolized and resynthesized to be incorporated into infant brain tissue (8).

Most HMOSs, including those containing Neu5Ac, however, seem to reach the colon where they are important growth factors for bifidobacteria, lactobacilli, and Bacteroides thetaiotaomicron, some of which are equipped with enzymatic systems to specifically metabolize HMOSs (9–13). The growth and metabolism of these bacteria provide energy and vitamins used by human enterocytes and contribute to the competitive exclusion of pathogens and maturation of the gut tissue (14–16).

A small portion of total HMOSs passes the intestinal epithelium and enters the circulation (17–19). On absorption, HMOSs can function as immune modulators through interference
with selectin-mediated cell-cell interactions (20–22). The effect of this may be an alleviated immune response and the prevention of a chronic inflammatory state (23, 24).

One liter of human milk contains ~20–23 g (colostrum) and 12–13 g (mature milk) free HMOSs (25, 26). This is ~10- to 100-fold the concentration of oligosaccharides found in cow milk (27, 28). The structural diversity within the oligosaccharide fraction in human milk exceeds that found in cow milk by far. All HMOSs have a core structure consisting of lactose at the reducing end variably elongated with fucosylated and/or sialylated lacto-N-biose units or N-acetyllactosamine units (26, 29–31). 1-fucose, Neu5Ac, or both can be found linked to the core without further elongation. As a result of multiple core structures and multiple linkage sites of each core, HMOSs exist in various isomeric forms. In considering all of the combinatorial possibilities of the identified compositional units, there theoretically could be >1000 different HMOSs (29, 32).

Today, the chemical structures of >100 HMOSs have been identified compared with ~40 HMOSs in cow milk (26, 27). Free oligosaccharides from milk of dairy species consist of shorter oligomeric chains and a different pool of mono- saccharide units than those of HMOSs (26, 33). For example, oligosaccharides containing the sialic acid variant Neu5Gc (N-glycolylneuraminic acid) are found in dairy milk and in milk of many other species including nonhuman primates, but not in human milk (27). Although HMOSs are highly fucosylated, free fucosyloligosaccharides in cow milk are rare. Glycan fucosylation in cow milk is mostly limited to core fucosylation of glycoprotein N-glycans (27, 29).

The evolutionary reasons for the striking differences in milk oligosaccharide quantity, structure, and complexity between mammalian species should be linked to interspecific differences in the neonates’ needs. One aspect of this causal relation is selective pressures imposed by variable exposure to pathogens. For example, it has been speculated that the human lack of Neu5Gc confers protection from animal-associated pathogenic strains of rotavirus that prefer to bind to this sialic acid (34). The HMOS 3′-sialyllactose is known to bind human-specific Helicobacter pylori (35). Fucosylated HMOSs may increase the human chances to ward off infective diarrhea caused by Campylobacter jejuni, which are harmless in cows (36–38).

All neonate mammals probably need milk stachic acids as building blocks for the developing brain, but the demand by human infants might be particularly high because of the exceptionally high brain:body mass ratio and high brain sialic acid concentration during all postnatal developmental stages (39).

However, differences in milk glycans not only exist between humans and other species but also between lactating women and within individual women between different time points of lactation (40, 41). It is also reasonable to assume that human-milk glycan variability is maintained by selective pressures that result from variable environmental challenges.

**HMOS-based human-milk types**

HMOS profile comparisons between mothers have shown a clear pattern concerning the absence or presence and concentration of fucosylated HMOs (31, 40, 42, 43). This pattern is largely determined by polymorphisms of 2 genes coding for fucosyltransferases: 1) fucosyltransferase 3 encoded by the Lewis gene adds fucose at subterminal position in α(1, 3)- and α(1, 4)-glycosidic linkage to the precursor HMOS substrate; 2) fucosyltransferase 2 encoded by the secretor gene adds fucose at terminal position in α(1, 2)-glycosidic linkage to the HMOS precursor (44, 45). Both genes have dominant functional alleles (Le and Se, respectively) and recessive alleles (le and se, respectively) with various single nucleotide polymorphisms rendering them nonfunctional (46, 47). Individuals with the allelic combination se/se (nonsecretors) do not express α(1, 2)-fucosyloligosaccharides in their body fluids, including saliva, urine, and milk (48).

The different allelic combinations of the Lewis and secretor genes yield 4 identifiable milk phenotypes: milk from women with the alleles Se/ — Le/ — (milk type 1) contains HMOSs that show α(1, 2)-, α(1, 3)-, and α(1, 4)-fucosylation. Milk from nonsecretor women possesses no α(1, 2)fucosylated HMOSs but does contain α(1, 3) fucosyloligosaccharides (se/se combined with le/le; milk type 4) or α(1, 3)-fucosyloligosaccharides and α(1, 4)-fucosylated HMOSs (se/se combined with Le/ — ; milk type 2). Milk type 3 (Se/ — with le/le) is characterized by the presence of α(1, 2)- and α(1, 3)-fucosylated HMOSs and the absence of α(1, 4)-fucosylated HMOSs (40). The total HMOS concentration in some of these milk types is also different; the HMOS concentration of milk type 3 tends to be higher than that of the other milk types (40, 49, 50). The clinical relevance of these findings, especially with regard to the risk of necrotizing enterocolitis for preterm-born infants, is a matter of ongoing discussions (50, 51).

In addition to these 4 milk types there may be many more polymorphisms in genes encoding HMOS-catalyzing enzymes that translate into more HMOS profile phenotypes or subtypes (52). Even with consideration of this hidden human-milk glycan diversity, the 4 Lewis/secretor HMOS types can be expected to be of paramount importance for defining the infant’s resistance to disease-impaired development in a given environment (53). This notion is supported by the fact that the Lewis/secretor HMOSs are identical in structure to epitopes of glycoconjugates on cell surfaces and soluble in body fluids similar to histo-blood group antigens (53). Lewis/secretor milk typing based on HMOSs shows close correlation with Lewis/secretor blood group typing via classical serology (40, 43).

Some histo-blood group types are known to affect infection risk, because pathogens can bind to type-specific antigen epitopes, whereas other epitopes are not recognized (54). For example, nonsecretors lack mucosal and epithelial binding sites for certain calcivirus strains; hence, they are less susceptible or even resistant to calcivirus-induced diarrhea (55, 56). However, nonsecretors may be at higher risk of infection with other pathogens, including bacterial strains of Escherichia coli that cause urinary tract infections and of Haemophilus influenzae that infect the upper respiratory tract (57, 58).

HMOSs that contain α(1, 2)-linked fucose inhibit host cell attachment by C. jejuni, common strains of calciviruses, and heat-stable enterotoxin of E. coli (36, 37, 59). These molecular effects can translate into lower risks of symptomatic infections in breastfed infants (36).

Maternal milk has long been known to be an important modulator of the child’s infection risk. Yet, the level of HMOS-induced protection against pathogens offered to a breastfed infant is the result of a complex and not yet fully understood
interplay between maternal genotype, infant genotype (which is not entirely independent of maternal genotype), and the infant’s degree of exposure to a given set of pathogens, among others.

**HMOS variability across lactation**

Pathogen distribution around the globe is nonuniform, with certain infection risks being markedly higher in some geographic regions than in others. It would be interesting to link the frequencies of the different human-milk types across multiple regions to regional infection risks (42, 60).

Variable exposure to pathogens may also be the evolutionary driver of quantitative and qualitative changes in HMOSs and in their lipid and protein conjugates during different stages of lactation and infant development, respectively. For the infant who is born with an immature immune system, the transition from the nearly sterile womb into a germ-rich environment marks the first major pathogen challenge. Another major transition that is linked with changed exposure to pathogens within the recommended breastfeeding period is the introduction of complementary food with the associated “new” pathogens at 4–6 mo of age. Here again, free and bound glycans from maternal milk may alleviate the risk of severe infections. This hypothesis has not yet been rigidly tested, but evidence for dynamic nonrandom milk-glycan changes during the lactational period is nevertheless growing.

For example, the total free HMOS concentration and the concentration of sialylated HMOSs both are much higher in colostrum than in mature human milk and steadily decline during extended lactation, with different HMOSs showing different dynamics (40, 49, 61, 62). In a study on colostrum from 12 Japanese women, the concentrations of 2 neutral HMOSs, 2’-fucosyllactose and lactodifucotetraose, on postpartum day 1 were detected to be substantially higher than those on days 2 and 3. In the same study, the lacto-N-tetraose concentration was found to increase in colostrum from postpartum day 1 to postpartum day 3 (61). In the milk of secretor women, the total concentration of α(1, 2)-fucosyloligosaccharides showed a significant decrease over the first 3 mo of lactation; α(1, 4)-fucosylated HMOS concentration in mature milk peaks in the first lactational month (40).

**GLYCOSOLIPIDS IN HUMAN MILK**

Milk glycolipids are almost exclusively located in the outer part of the lipid triple layer of the milk fat globule membrane (MFGM), where the oligosaccharide part may interact with the external environment, whereas the ceramide lipid chain functions structurally as an anchor (63). These glycolipids occur mainly in the form of glycosphingolipids. With concentrations of 1–20 mg/L milk, the Neu5Ac-containing gangliosides are the most abundant among them (64–68). Cow milk has a significantly lower content of gangliosides than does human milk (69, 70).

Gangliosides are incorporated in cell membranes of virtually all body organs, from intestinal epithelium to the central nervous system and play a central role in cell-cell communication, cell-matrix interactions, and growth and differentiation of cells, especially neurons (5, 71–73).

Dietary gangliosides can be absorbed in the small intestine and transported to different parts of the body or might function as prebiotic molecules (5, 66, 74, 75). Dietary uptake of gangliosides is reflected in the concentration of gangliosides in the mucosa, the plasma, and the brain (71, 76).

**Structure, distribution, and dynamic expression of gangliosides**

A number of gangliosides in human milk have recently been characterized, with monosialoganglioside (GM) 3 and disialoganglioside (GD) 3 as the most abundant types (66, 69, 77–79). The overall content of gangliosides in human milk follows a trend reported for other sialoglycoconjugates, with high values in colostrum and a decrease during subsequent lactation (80). GD3 concentration in human milk is highest at the beginning of lactation. GM3, in turn, shows an inverse pattern with low initial concentrations and a continuous rise during the course of lactation (74, 77, 79, 81, 82). Recent studies have focused on structural differences between cow- and human-milk gangliosides and on functional aspects of dynamic expression (5, 63, 83–85).

**Influence of gangliosides on the immune system**

After ingestion and absorption into the intestinal epithelium, dietary GM3 and GD3 show different distribution. GD3 accumulates selectively in the basolateral region of the intestinal epithelial cells, whereas GM3 predominates in the apical region (76). In vitro, cow-milk–derived GM3 and GD3 have been shown to differentially influence dendritic cell maturation and downstream effects, suggesting a role in the early postnatal modulation of oral tolerance toward nonaggressive antigens (66, 86). Because of the higher concentration of GD3 in early-lactation milk, immune-stimulating effects may be strongest during this period. In a model of infant necrotizing enterocolitis, gangliosides exerted a protective antiinflammatory effect by modulating vasoactive mediators and by suppressing proinflammatory signals (75). Dietary supplementation with gangliosides in other animal studies induced a positive stimulation of the intestinal immune system by affecting the proliferation of cytokine-secreting lymphocytes and the production of IgA-secreting cells of the small intestine (66). In summary, human-milk gangliosides may have a role in the healthy development of the neonatal intestinal immune system.

**Antiflammatory properties of gangliosides**

Dietary sialic acid–containing gangliosides in the gut are likely to exert direct antipathogenic effects (66). The antipathogenic effect was first shown by the inhibition of enterotoxin from *E. coli* and *Vibrio cholerae* (78, 87–89). The minor milk ganglioside GM1 could later be identified as the specific antipathogenic agent (90). It has also been shown that human-milk lipids, and gangliosides in particular, can bind Shiga toxin (91). Other studies reported neutralizing effects of human-milk gangliosides toward cholera toxin, botulinum neurotoxin type A, and *H. pylori* vacuolating cytotoxin, even in very small amounts (92, 93). In piglets, dietary GM1 and the GM3 were shown to act as decoy receptors for porcine rotavirus (94). Sulfated glycolipids from human milk were found to inhibit HIV-1 infection in vitro (95). Antiviral effects have also been described for GM3 and GD3 on the influenza A virus as well as for GD3 from bovine milk on the influenza C virus (96, 97). Moreover,
GLYCOPROTEINS IN HUMAN MILK

Human milk contains a wide variety of proteins that contribute to its unique qualities (102). Many of the human-milk-proteins are easily digested, thus providing a balanced amino acid composition that is utilizable as building blocks for healthy growth by the infant. In addition to these nutritive proteins, human milk also contains numerous functional proteins, which are more resistant to proteolysis in the gastrointestinal tract to preserve their functionality throughout their transit through the stomach and small intestine (103–105). Both resistance to digestive enzymes and structure-function relations of proteins are modulated by glycosylation—ie, by covalent attachment of oligosaccharide chains to proteins via threonine or serine (O-glycosylation) or asparagine (N-glycosylation) (104, 106). Glycosylation is a common posttranslational protein modification that affects most secreted and membrane-bound proteins, including some major milk proteins (107–110). However, the amount of glycosylated proteins as a percentage of the total protein content as well as the number and monosaccharide makeup of oligosaccharide chains differ widely between mammalian species and, for example, between human milk and cow milk (111–113). In comparison, for pathogenic gastrointestinal bacteria, viruses, and yeasts (10, 117, 118). Multiple glycosylation sites at single protein macromolecules lead to an increased potency of these effects; however, this is not the only way in which the antipathogenic action of glycoproteins is achieved.

Function of human-milk glycoproteins

Similar to free HMOs, to which they are structurally related, the glycan epitopes of human-milk glycoproteins such as mucins function as prebiotic factors in the formation and stabilization of a favorable intestinal microbiota and as effective decoy receptors for pathogenic gastrointestinal bacteria, viruses, and yeasts (10, 117, 118). Multiple glycosylation sites at single protein macromolecules lead to an increased potency of these effects; however, this is not the only way in which the antipathogenic action of glycoproteins is achieved.

The glycoprotein lactoferrin acts as a specific iron (Fe3+) chelator (119). By selective removal of iron from the medium, microbial growth can be inhibited (120–122).

Peptides that result from the enzymatic break-down of glycoproteins such as lactoferricin can also display antibacterial and antiviral properties (123–125). Some inhibit pathogen growth in the absence of inflammation, whereas others are most active in an inflamed environment (123).

Evidence that suggests a role in the defense against pathogens is available for a number of prominent glycoproteins in human milk. In addition to mucins (126) and lactadherin (127, 128), 2 glycoproteins of the milk MFGM, these are lactoferrin (122, 128, 129), α-lactalbumin (130), bile salt–stimulated lipase (BSSL) (131), secretory IgA (sIgA) (132), κ-casein (130), lactoperoxidase (111), and osteopontin (128, 133). Antipathogenic mechanisms of glycoproteins include interactions with immune cells involved in the innate and adaptive immune responses (36, 122, 127, 129, 134).

Other biological functions of human-milk glycoproteins in the neonate relate to organ development such as intestinal mucosa, brain, bones, and teeth (99, 102, 133, 135); enhanced nutrient uptake (iron and calcium) (102, 111, 130, 136, 137); and improved fat digestion (138).

Quantitative and qualitative variability of human-milk glycoproteins

Both the expression and glycosylation of glycoproteins in human milk can vary throughout lactation. Quantitative variation in the expression of glycoproteins has been reported for κ-casein (102), BSSL (109), sIgA (109, 139, 140), lactoferrin (109, 141, 142), lactoperoxidase (143), and osteopontin (144) and for glycoproteins of the MFGM such as lactadherin (118, 145). Concentrations of sIgA in human milk, for instance, reach a maximum immediately after delivery and then decline markedly within a few days (109, 139, 140). In comparison, α-lactalbumin, which is highly abundant but seldom (~1%) glycosylated (146), and mucins are more constant (102).

With regard to the degree of glycosylation, no changes have yet been detected for sIgA (109). However, evidence for dynamic changes is available for lactoferrin and BSSL (109, 110, 147–149).

For BSSL a dynamic pattern of glycosylation has been found during the first 10 d of lactation (109, 149). Whereas the concentration of BSSL remains more or less constant during this period, the abundance of glycans is approximately doubled and
their structure changed (109). Over the course of 6 mo of lactation, a decrease in both total carbohydrate content and relative amount of sialic acid and an increase in fucosylated structures were shown (147). During late lactation, BSSL was found to eventually be replaced by a completely nonglycosylated analog (109).

For human-milk lactoferrin, a decrease in total glycosylation by ~60% was observed between days 2 and 5 of lactation followed by an increase in glycosylation to near its previous levels at day 15 (109). In addition, an increase in bifucosylated complex glycans throughout lactation was observed. These changes were accompanied by altered expression of oligosaccharyl- and fucosyltransferases in milk somatic cells (149). Similar glycosylation dynamics may happen over smaller time scales.

The identification of 52 N-linked glycans in 3 sampled women provided preliminary information on the range of the diversity of N-glycosylation. Eighty-four percent of the N-glycans were fucosylated and 47% sialylated; 70% of total N-glycan abundance was composed of N-glycans found in all 3 milk samples (150).

The prevalence of fucose in human-milk N-glycans may exert antipathogenic effects similar to fucose units in free HMOs (150). Sialic acid in human milk is an essential nutrient for infant brain development and cognition (99). More than 20% of the sialic acid in human milk is derived from glycoproteins (80). However, it is currently not known which source of sialic acid—HMOs, gangliosides, or glycoproteins—is the most important for brain sialic acid (150).

Dynamic human-milk protein glycosylation can potentially regulate proteolytic susceptibility, pathogen binding, prebiotic effects, and participation in various other developmental processes in the infant (149, 151). However, precisely how variable human-milk protein glycosylation can drive the healthy development of the neonate is still almost completely unknown (109, 149).

CONCLUSIONS

Carbohydrates and their lipid and protein conjugates are a major class of free and lipid membrane (eg, MFGM)–anchored human-milk compounds. They serve the nutritional needs of human infants as a direct source of energy (lactose), building blocks for healthy growth (sialic acids, gangliosides), mediators of immune cell functioning (HMOs entering circulation), food for beneficial gut microbes, direct antipathogenic defense (free and bound HMOs), and regulators of milk protein function and digestion (protein-bound glycans). On the structural level, human-milk glycans offer tremendous potential for diverse interactions with receptor molecules aimed at producing many specific effects that benefit the infant. Notable aspects of substantial complexity related to their function include the temporal changeability of glycan appearance in milk and the linkage of certain glycoepitopes present in milk with glycoepitope expression in other body fluids and tissues via specific genes. The usefulness of complex milk glycostructures for the infant and the ability to secrete them are constrained by the infant’s and mother’s genotype, respectively, as well as by geographic features. A common theme of these aspects that allows the formulation of testable predictions for guidance of future research is pathogen defense. Pathogens are a major threat to infant health and hence powerful selective agents. A better understanding of the precise contribution of dietary milk glycans for achieving pathogen strain–specific immunity in the infant in an evolutionary response to this selective pressure should be one of our central aims.

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