Human Milk Oligosaccharides: Every Baby needs a Sugar Mama

Lars Bode

Division of Neonatology and Division of Gastroenterology and Nutrition, Department of Pediatrics, University of California, San Diego, CA, USA

Corresponding Author: Lars Bode, PhD
Assistant Professor of Pediatrics
University of California, San Diego
Department of Pediatrics
Division of Neonatology and
Division of Gastroenterology and Nutrition
200 West Arbor Drive, MC 8450
San Diego, CA 92103-8450
phone: (619) 543-7545
fax: (619) 543-7537
lbode@ucsd.edu
ABSTRACT

Human Milk Oligosaccharides (HMO) are a family of structurally diverse unconjugated glycans that are highly abundant in and unique to human milk. Originally, HMO were discovered as prebiotic “bifidus factor” that serves as metabolic substrate for desired bacteria and shapes an intestinal microbiota composition with health benefits for the breast-fed neonate. Today, HMO are known to be more than just “food for bugs”. An accumulating body of evidence suggests that HMO are antiadhesive antimicrobials that serve as soluble decoy receptors, prevent pathogen attachment to infant mucosal surfaces and lower the risk for viral, bacterial and protozoan parasite infections. In addition, HMO may modulate epithelial and immune cell responses, reduce excessive mucosal leukocyte infiltration and activation, lower the risk for necrotizing enterocolitis, and provide the infant with sialic acid as a potentially essential nutrient for brain development and cognition. Most data however, stems from in vitro, ex vivo or animal studies, and occasionally from association studies in mother-infant cohorts. Powered, randomized and controlled intervention studies will be needed to confirm relevance for human neonates. The first part of this review introduces the pioneers in HMO research, outlines HMO structural diversity, and describes what is known about HMO biosynthesis in the mother’s mammary gland and their metabolism in the breast-fed infant. The second part highlights the postulated beneficial effects of HMO for the breast-fed neonate, compares HMO to oligosaccharides in the milk of other mammals and in infant formula, and summarizes the current roadblocks and future opportunities for HMO research.

Nutrition / Dietary Glycans / Prebiotics / Infections / Inflammation
Origins of HMO Research

The discovery of Human Milk Oligosaccharides (HMO) was driven by scientists and physicians with two very different perspectives and interests. Pediatricians and microbiologists were trying to understand the observed health benefits associated with human milk feeding. Chemists were trying to characterize the highly abundant carbohydrates uniquely found in human milk.

Already at the end of the 19th century, when overall infant first-year mortality rates were as high as 30%, it was observed that breast-fed infants had a much higher chance of survival and had lower incidences of infectious diarrhea and many other diseases than "bottle-fed" infants. At that time Theodore Escherich, an Austrian pediatrician and microbiologist, had just discovered a relationship between intestinal bacteria and the physiology of digestion in infants (Escherich 1886). Guided by observations that infant health is linked to both breast-feeding and intestinal bacteria, Ernst Moro, one of Escherich’s former students, and Henri Tissier, a graduate student in Paris, independently found differences in the bacterial composition in the feces of breast-fed compared to “bottle-fed” infants (Moro 1900, Tissier 1900). Which components in human milk determined the bacterial composition in the infant’s intestine remained unknown until more than half a century later.

In parallel to the observations by pediatricians and microbiologists, the chemists’ interest in HMO evolved when Eschbach noted in 1888 that human milk contained “a different type of lactose” than bovine milk (reviewed in (Montreuil 1992). Shortly after, Deniges found that lactose in human and bovine milk is the same but that human milk contains an additional unknown carbohydrate fraction (reviewed in (Montreuil 1992). In the early 1930th, more than 40 years later, Michel Polonowski and Albert Lespagnol were able to characterize this carbohydrate fraction and called it “gynolactose” (Polonowski 1929, Polonowski 1930,
Polonowski 1931, Polonowski 1933). It was only weakly soluble in methanol, contained nitrogen and hexosamines, and consisted of various components. A few years later, Polonowski and Jean Montreuil introduced two-dimensional paper chromatography to separate “gynolactose” into individual oligosaccharides (Polonowski 1954). The exact structures and potential functions of these oligosaccharides were however unknown.

HMO research experienced a breakthrough when chemist Richard Kuhn and pediatrician Paul György, one of Moro’s former students, started to collaborate. Earlier, in 1926, Herbert Schönfeld had reported that the whey fraction of human milk contains a growth-promoting factor for *Lactobacillus bifidus* (later reclassified as *Bifidobacterium bifidus*) (Schönfeld 1926). The chemical nature of the “bifidus factor” in human milk was unknown, but Kuhn and György hypothesized a connection between Moro’s and Tissier’s work on bacteria and Polonowski’s and Lespagnol’s work on “gynolactose”. In the end, they were able to confirm that the “bifidus factor” indeed consists of oligosaccharides (Gauhe *et al.* 1954, György 1954, György *et al.* 1954a, György *et al.* 1954b, Rose *et al.* 1954).

In the following years, Montreuil’s group in France and Richard Kuhn’s group in Germany discovered and characterized more than a dozen individual HMO (Kuhn 1956a, Kuhn 1956b, Kuhn 1958b, Kuhn 1958a, Kuhn 1960, Montreuil 1960, Kuhn 1962). Some of the oligosaccharides showed activities of blood group determinants, which contributed to the structural characterization of H and Lewis blood group determinants (Grollman *et al.* 1967, Grollman *et al.* 1969, Grollman *et al.* 1970, Kobata 2010). In return, the interest in blood group glycans led to the development of new methods and tools that supported the discovery and characterization of many additional HMO with Victor Ginsburg and Akira Kobata as some of the most influential investigators (Kobata *et al.* 1969a, Kobata *et al.* 1969b, Kobata *et al.* 1972a, Kobata *et al.* 1972b, Kobata 2010). Heinz Egge, one of Kuhn’s former students in Germany,
was one of the first to use fast atom bombardment mass spectrometry to characterize additional HMO (Egge et al. 1983), and his former student, Clemens Kunz, a nutritional scientist, continues to study the nutritional and biological properties of HMO (Kunz et al. 1996, Rudloff et al. 1996, Gnoth et al. 2000, Gnoth et al. 2001, Rudloff et al. 2002, Bode et al. 2004a, Bode et al. 2004b, Rudloff et al. 2006, Kuntz et al. 2008, Kuntz et al. 2009, Rudloff et al. 2011). Figure 1 shows a timeline with key events in HMO discovery until the end of the 20th century.

Concentration, Composition and Variation

Oligosaccharide amount and composition vary between women and over the course of lactation (reviewed in (Kunz et al. 2000). Colostrum, the thick, yellowish fluid secreted by the mammary gland a few days before and after parturition, contains as much as 20-25 g/L HMO (Coppa et al. 1999, Gabrielli et al. 2011). As milk production matures, HMO concentrations decline to 5-20 g/L (Coppa et al. 1999, Kunz et al. 1999, Newburg et al. 2000, Chaturvedi et al. 2001a, Davidson et al. 2004, Bao et al. 2007, Gabrielli et al. 2011), which still exceeds the concentration of total milk protein. The milk of mothers delivering preterm infants has higher HMO concentrations than term milk (Gabrielli et al. 2011). The wide range in HMO concentrations reflects interpersonal variations as well as differences in the non-standardized analytical methods used in various laboratories. Table 1 lists macromolecule concentrations in mature human milk and compares it to bovine milk, the basis of most infant formula.

HMO are composed of the five monosaccharides glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia), with N-acetylneuraminic acid (Neu5Ac) as the predominant if not only form of Sia. HMO biosynthesis appears to follow a basic blueprint (Figure 2A): All HMO contain lactose (Galβ1-4Glc) at their reducing end, which can be elongated by the addition of β1-3- or β1-6-linked lacto-N-biose (Galβ1-3GlcNAc-, type 1
chain) or N-acetyllactosamine (Galβ1-4GlcNAc-, type 2 chain). Elongation with lacto-N-biose appears to terminate the chain, whereas N-acetyllactosamine can be further extended by the addition of one of the two disaccharides. A β1-6 linkage between two disaccharide units introduces chain branching. Branched structures are designated as iso-HMO; linear structures without branches as para-HMO (Figure 2B). Lactose or the elongated oligosaccharide chain can be fucosylated in α1-2, α1-3 or α1-4 linkage and/or sialylated in α2-3 or α2-6 linkage (Figure 2C-E). Some HMO occur in several isomeric forms, e.g. lacto-N-fucopentaose (LNFP, Figure 2D) or sialyllacto-N-tetraose (LST, Figure 2E).

More than a hundred different HMO have been identified so far, but not every woman synthesizes the same set of oligosaccharides (reviewed in (Kobata 2010)). HMO composition mirrors blood group characteristics, which depend on the expression of certain glycosyltransferases. Four milk groups can be assigned based on the Secretor and Lewis blood group system, which is determined by the activity of two gene loci encoding for the α1-2-fucosyltransferase FUT2 (encoded by the Se gene) and the α1-3/4-fucosyltransferase FUT3 (encoded by the Le gene) (Kumazaki et al. 1984, Viverge et al. 1990, Johnson et al. 1992, Thurl et al. 1997, Chaturvedi et al. 2001a, Stahl et al. 2001, Thurl et al. 2010). Individuals with an active Se locus are classified as Secretors. Milk of Secretor women is abundant in 2’-fucosyllactose (2’FL), lacto-N-fucopentaose 1 (LNFP1) and other α1-2-fucosylated HMO. In contrast, Nonsecretors lack a functional FUT2 enzyme and their milk does not contain α1-2-fucosylated HMO. Individuals with an active Le locus are classified as Lewis positive. They express FUT3, which transfers Fuc in α1-4 linkage to subterminal GlcNAc on type 1 chains (Xu et al. 1996). In contrast, the milk of Lewis negative women lacks these specific α1-4-fucosylated HMO, e.g. lacto-N-fucopentaose 2 (LNFP2). Figure 3 shows that breast milk can be assigned to one of four groups based on the expression of FUT2 and FUT3: Lewis-positive Secretor
(Se+Le+), Lewis-negative Secretor (Se+Le-), Lewis-positive Nonsecretor (Se-Le+) and Lewis-negative Nonsecretor (Se-Le-). The classification of individual milk samples into these four groups is however an oversimplification of HMO complexity. For example, FUT2 and FUT3 compete for some of the same substrates (Kumazaki et al. 1984, Johnson et al. 1992, Xu et al. 1996) and the levels of enzyme expressions and activities create a continuum of HMO profiles throughout the population. Even the milk of Lewis-negative Nonsecretor women that express neither FUT2 nor FUT3 contains fucosylated HMO like 3-fucosyllactose (3FL) or lacto-N-fucopentaose 3 (LNFP3), suggesting that other, Se- and Le-independent fucosyltransferases (FUT4, 5, 6, 7 or 9) may be involved (Newburg et al. 2005). In addition, α1-2-fucosylated HMO have been found in the milk of Nonsecretor women towards the end of lactation, and Newburg et al. suggested that FUT1 may also participate in HMO fucosylation (Newburg et al. 2005).

In addition to these genetic variations, other factors such as nutritional and environmental aspects may also affect oligosaccharide amount and composition, but there is currently no data to support these speculations.

**Biosynthesis**

Since all HMO carry lactose at their reducing end, it is likely that HMO biosynthesis is an extension of lactose biosynthesis, which occurs in the Golgi and starts with Glc (Figure 4). Some of the cytosolic glucose is activated to UDP-Glc and converted to UDP-Gal. Studies with 15C-labeled Gal suggest that exogenous dietary Gal might be directly incorporated into lactose and HMO without prior conversion to Glc in the liver and reconversion to Gal in the mammary gland (Rudloff et al. 2006). Eventually, both UDP-Gal and Glc are transported into the Golgi and are linked by the lactose synthase complex that consists of two components: The “A” protein is a constitutively expressed β1-4 galactosyltransferase (β4GalT1) that by itself transfers UDP-Gal
to terminal GlcNAc during glycoconjugate biosynthesis. In combination with the “B” protein \( \alpha \)-lactalbumin, which is expressed under the control of lactation hormones, \( \beta 4 \)GalT1 shifts its acceptor specificity from GlcNAc to Glc to yield lactose (Ramakrishnan et al. 2002).

How lactose is extended to form the different HMO remains poorly understood. As described in a later section of this review, milk oligosaccharide amount and composition highly vary between species, which makes it difficult to study HMO biosynthesis in animal models. A battery of glycosylation knockout mice would be available to investigate which parts of the glycosylation machinery are involved in milk oligosaccharide biosynthesis. Unfortunately, mouse milk contains only 3'- and 6'-sialyllactose, but none of the elongated, branched or fucosylated oligosaccharides found in human milk (Fuhrer et al. 2010). Others and we have used immortalized or transformed human mammary gland epithelial cells to study HMO biosynthesis, with limited success. None of the cell lines synthesized HMO, not even lactose. Most of them did not even express \( \alpha \)-lactalbumin, essential for lactose synthesis. This lack of suitable models limits our current understanding of which enzymes and transporters participate in HMO biosynthesis and how it is regulated.

Kobata suggested that HMO elongation and branching follows the example of poly-N-acetyllactosamine synthesis on glycoconjugates and that the lactose core is extended by alternating actions of N-acetylglucosaminyltransferases (GlcNAcT) and galactosyltransferases (GalT) (Kobata 2003). A \( \beta 3 \)GlcNAcT (iGnT) would initiate linear chain elongation, corresponding to the “i” blood group antigen. A \( \beta 6 \)GlcNAcT (IGNT) would initiate chain branching, corresponding to the “I” antigen. A \( \beta 3 \)GalT would produce a type 1 disaccharide unit (Gal\( \beta 1-3 \)GlcNAc-), while a \( \beta 4 \)GalT would generate a type 2 disaccharide unit (Gal\( \beta 1-4 \)GlcNAc-). Based on our current knowledge in glycan synthesis, it seems likely that these enzymes are involved in
HMO elongation and branching. However, direct evidence to support this hypothesis is currently not available.

HMO fucosylation is fairly well understood and has been elucidated based on the involvement of blood group-synthesizing fucosyltransferases as outlined above. Fucosyltransferase 2 (FUT2) adds fucose in an α1-2 linkage to terminal Gal, and fucosyltransferase 3 (FUT3) adds fucose in an α1-4 linkage to internal GlcNAc, preferentially on type 1 chains. Expression of FUT2 and FUT3 strictly depends on the activity of the Se and Le gene loci (Kumazaki et al. 1984, Johnson et al. 1992, Chaturvedi et al. 2001a, Stahl et al. 2001). As mentioned in the previous section on HMO composition, an additional unknown fucosyltransferase (FUTx in Figure 4) is Se- and Le-independent and adds fucose in α1-3 linkage to the reducing end Glc or internal GlcNAc of type 2 chains. Thus, even the milk of Lewis-negative Nonsecretor (Se-Le-) women contains some fucosylated HMO, e.g. 3-FL or LNFP3 (Thurl et al. 1997, Chaturvedi et al. 2001a, Stahl et al. 2001, Thurl et al. 2010, Gabrielli et al. 2011). While mouse milk contains only 3’- and 6’-sialyllactose and no fucosylated oligosaccharides, 2’-fucosyllactose appears in the milk of transgenic mice that express FUT1 under the control of a lactogenic promoter (Prieto et al. 1995).

Several questions remain unanswered with respect to HMO sialylation. Analyzing the milk of β-galactoside sialyltransferase (STGal) knockout mice revealed that ST3Gal4 and ST6Gal1 are involved in the biosynthesis of 3’-sialyllactose and 6’-sialyllactose, respectively (Fuhrer et al. 2010). Whether these two transferases also sialylate the terminal Gal in complex milk oligosaccharides found in human milk remains unknown. In addition, the subterminal GlcNAc of HMO can be sialylated in α2-6 linkage, which is a rather unusual structural feature and rarely found on human tissues with the exception of the central nervous system and certain tumors.
Six different ST6GalNAc have been identified in humans, but not a single ST6GlcNAc. In certain colon cancer lines ST6GalNAc5 facilitates α2-6-sialylation of subterminal GlcNAc (Tsuchida et al. 2003). However, the terminal Gal needs to be α2-3-sialylated before ST6GalNAc5 adds sialic acid to the subterminal GlcNAc. Asialo-LNT is not a substrate for ST6GalNAc5. Human milk contains disialyllacto-N-tetraose (DSLNT), which is α2-3-sialylated at the terminal Gal and would qualify as ST6GalNAc5 substrate. However, human milk also contains LST b, which contains α2-6-sialylated subterminal GlcNAc, but the terminal Gal is not sialylated, which would exclude the involvement of ST6GalNAc5 based on previous reports on substrate specificity (Tsuchida et al. 2003). Which transferases contribute to the sialylation of DSLNT and LST b remains to be identified. In general, our knowledge on HMO biosynthesis remains fairly limited. Novel, more suitable models are needed to help close this gap.

**Metabolism**

Once ingested, HMO resist the low pH in the infant’s stomach as well as digestion by pancreatic and brush border enzymes based on data from *in vitro* degradation studies (Engfer et al. 2000, Gnoth et al. 2000). Data obtained in the 1980s and 90s shows that HMO reach the distal small intestine and colon in an intact form and are excreted with the infant’s feces (Sabharwal et al. 1984, Sabharwal et al. 1988a, Sabharwal et al. 1988b, Sabharwal et al. 1991, Chaturvedi et al. 2001b, Coppa et al. 2001). More recent studies with capillary electrophoresis and laser induced fluorescence detection and mass spectrometry confirm and refine these original observations and suggest multi-stage HMO processing and degradation that depend on infant age, blood group and feeding regime (Albrecht et al. 2010, Albrecht et al. 2011a, Albrecht et al. 2011b). In the first stage between birth and about two months of life, feces of breast-fed infants contains sialylated and non-sialylated HMO that are similar, but not identical to the corresponding milk
samples. In the subsequent second stage, the feces contains mainly HMO degradation and processing products that are fairly different from the HMO in the corresponding milk samples. In the third stage, starting from when feedings other than human milk are introduced, HMO entirely disappear from the infant’s feces (Albrecht et al. 2011a).

Rudloff et al. were the first to show that intact HMO appear in the urine of preterm breast-fed infants, but not in formula-fed infants (Rudloff et al. 1996). These results suggest that HMO are absorbed in the infant’s intestine and reach the systemic circulation. In the following years, the same group used a set of elegant and elaborate *in vivo* $^{13}$C-labeling studies to further investigate HMO metabolism (Obermeier et al. 1999, Rudloff et al. 2006, Rudloff et al. 2011). Lactating women received an oral bolus of $^{13}$C-labeled Gal and their mammary glands incorporated the label during HMO synthesis. The breast-fed infant then ingested the *in vivo*-labeled HMO with the mother’s milk and approximately 1% appeared in the infant’s urine. In fact, the label was still at the same position as in the $^{13}$C-label of the orally administered Gal, indicating direct incorporation and minimal rearrangement.

While non-sialylated HMO cross monolayers of cultured intestinal epithelial cells by receptor-mediated transcytosis and paracytosis, sialylated HMO use paracytosis only (Gnoth et al. 2001). It remains unknown what receptors facilitate absorption and how rapid HMO are absorbed, appear in the circulation, and are cleared from the system.

**Postulated Beneficial Effects**

Originally identified as the “bifidus factor” in human milk, HMO had mostly been recognized for their “bifidogenic” or prebiotic effects. However, since the early 1990s, accumulating evidence suggests that HMO are more than just a substrate to promote the growth of desired bacteria in the infant’s intestine. The following sections outline the postulated effects of HMO on breast-fed
infants and potentially also on breast-feeding mothers. Figure 5 illustrates some of the discussed effects.

**Prebiotics**

Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host well-being and health” (Gibson *et al.* 2004, Roberfroid 2007a). This definition requires that prebiotics are resistant to gastric acidity, hydrolysis by host enzymes, and gastrointestinal absorption. HMO meet all three criteria given that an absorption rate of ~1% can be neglected in this specific context and that the great majority of HMO reach the distal small intestine and colon intact and at high concentrations. *B. longum* subsp *infantis* (*B. infantis*, JCM1222) grows well when HMO are offered as the only carbohydrate source (LoCascio *et al.* 2007, Marcobal *et al.* 2010, Asakuma *et al.* 2011). Over time, *B. infantis* consumes HMO entirely, including mono- and disaccharide degradation products (Asakuma *et al.* 2011). Sequencing of the *B. infantis* genome revealed entire gene clusters that control the expression of specific glycosidases, sugar transporters and glycan binding proteins dedicated to HMO utilization (Sela *et al.* 2008). Compared to *B. infantis*, *B. bifidum* (JCM1255) grows slightly slower on HMO and leaves behind at least some of the monosaccharide degradation products (Asakuma *et al.* 2011). In contrast, *B. longum* subsp. *longum* (JCM1217) and *B. breve* (JCM1192) hardly grow on HMO at all and metabolize only LNT (Galβ1-3GlcNAC-Lac, type I), but not LNnT (Galβ1-4GlcNAc-Lac, type 2). Hence, it is incorrect to refer to HMO as “bifidogenic” since they promote the growth of certain, but certainly not all Bifidobacteria.

While most data on prebiotic effects of HMO stem from isolated *in vitro* fermentation studies, a most recent report in germ-free mice showed that LNnT provides a significant growth advantage for *B. infantis* over co-inoculated *Bacteroides thetaiotaomicron* (Marcobal *et al.* 2011). *In vitro,*
*B. infantis* grows well on LNnT as the only carbohydrate source; *B. thetaiotaomicron* does not. In germ-free mice that are co-inoculated with *B. infantis* and *B. thetaiotaomicron*, the relative abundance of *B. infantis* is only around 2% when LNnT is absent from the diet, but increases to over 40% when LNnT is introduced with the drinking water. It remains to be investigated how these results translate to an environment with more complex bacterial communities and the presence of other bioactive milk compounds in human neonates.

The HMO-mediated dominance of *B. infantis* may keep potentially harmful bacteria in check as they compete for limited nutrient supply. In addition, *B. infantis* and several other infant-associated bacteria produce short chain fatty acids and other metabolites (postbiotics) that create an environment favoring the growth of commensals over potential pathogens (Gibson *et al.* 1994)(Figure 5A).

Antiadhesive Antimicrobials

In addition to indirectly keeping pathogens in check by providing a competitive advantage to non-pathogenic commensals, HMO also directly reduce microbial infections by serving as antiadhesive antimicrobials (Kunz *et al.* 2000, Newburg *et al.* 2005). Many viral, bacterial or protozoan pathogens need to adhere to mucosal surfaces to colonize or invade the host and cause disease. Pathogen adhesion is often initiated by lectin-glycan interactions. For example, *Escherichia (E.) coli* with type 1 fimbriae bind to mannose-containing glycans while *E. coli* with S fimbriae as well as *Helicobacter pylori* bind to sialylated glycans (Firon *et al.* 1983, Parkkinen *et al.* 1983). Glycan-mediated attachment mechanisms have also been described for many viruses like noroviruses or rotaviruses (Hu 2012), which are among the most common causes of severe diarrhea in infants and young children and responsible for almost half a million deaths annually (Tate *et al.* 2012). Some HMO resemble mucosal cell surface glycans, serve as soluble decoy
receptors to prevent pathogen binding, and reduce the risk of infections (Simon et al. 1997, Gustafsson et al. 2006).

The most comprehensive data on HMO as antiadhesive antimicrobials has been reported for Campylobacter jejuni infections, which are one of the most common causes of bacterial diarrhea and infant mortality (Ruiz-Palacios et al. 2003, Morrow et al. 2004). C. jejuni binds to cultured epithelial cells via type 2 H-antigens, which are α1-2-fucosylated glycans (Fucα1-2Galβ1-4GlcNAc-R) (Ruiz-Palacios et al. 2003). Addition of soluble α1-2-fucosylated HMO blocks C. jejuni binding to cultured cells and human intestinal mucosa explants and reduces C. jejuni colonization in mice. The beneficial effect of α1-2-fucosylated HMO on reducing episodes of C. jejuni-associated diarrhea has been confirmed in a prospective study on almost 100 mother-infant pairs from a transitional neighborhood of Mexico City (Morrow et al. 2004). C. jejuni diarrhea occurred significantly less often in infants whose mother’s milk contained high concentrations of 2’-fucosyllactose. In addition, calicivirus diarrhea occurred less often in infants whose mother’s milk contained high concentrations of lacto-N-difucohexaose I, another α1-2-fucosylated HMO.

Instead of using lectins to bind to host glycans, some microorganisms express glycans that bind to lectins on host cells. For example, the envelope glycoprotein gp120 facilitates binding of human immunodeficiency virus (HIV) to DC-SIGN (Dendritic Cell-Specific ICAM3-Grabbing Non-integrin) on human dendritic cells that peek through mucosal surfaces and screen the environment for potential pathogens (Su et al. 2003, van Kooyk et al. 2003, Wu et al. 2006). The initial gp120/DC-SIGN interaction is important for HIV entry through mucosal barriers during HIV mother-to-child transmission via breast-feeding. While DC-SIGN binds to high-mannose type glycans on gp120 (Zhu et al. 2000, Doores et al. 2010), it has even higher affinities to Lewis
blood group antigens (Naarding et al. 2005, van Liempt et al. 2006). HMO contain Lewis blood group antigens and compete with gp120 for binding to DC-SIGN in vitro (Hong et al. 2009). In the breast-fed infant, mucosal surfaces are covered with high concentrations of HMO, which may block HIV entry via DC-SIGN. This may explain why HIV mother-to-child transmission through breast-feeding is rather inefficient with 80-90% of infants not acquiring infections despite continuous exposure to the virus in milk over many months (Coutsoudis et al. 2004). This hypothesis has been further strengthened by HMO analysis from milk samples collected as part of a larger study of HIV-infected women and their infants followed from birth to 24 months in Lusaka, Zambia (Kuhn et al. 2008). Higher HMO concentrations in the mother’s milk were indeed associated with protection against postnatal HIV transmission independent of other known risk factors (Bode et al. manuscript in preparation).

Antiadhesive antimicrobial effects may not be restricted to bacteria and viruses; they might also apply to certain protozoan parasites like Entamoeba histolytica, which causes amoebic dysentery or amoebic liver abscess (Pritt et al. 2008). Worldwide, approximately 50 million people are infected with E. histolytica, resulting in nearly 100,000 deaths annually and making it the third leading cause of death by parasitic diseases, surpassed only by malaria and schistosomiasis (Pritt et al. 2008). E. histolytica colonization and invasion requires the attachment to the host’s colonic mucosa. Parasites that cannot attach are carried downstream and are excreted with the feces without causing disease. One of E. histolytica’s major virulence factors is the Gal/GalNAc-lectin, which facilitates parasite attachment as well as the killing and phagocytosis of intestinal epithelial cells (Cano-Mancera et al. 1987, Saffer et al. 1991a, Saffer et al. 1991b). Some HMO significantly reduce E. histolytica attachment and cytotoxicity in co-cultures with human intestinal epithelial cell lines (Jantscher-Krenn et al. 2012). In sharp contrast to C. jejuni infections, α1-2-fucosylated HMO have no effect on E. histolytica attachment and cytotoxicity, suggesting that a non-fucosylated terminal Gal on HMO is
important to bind to the Gal/GalNAc lectin. Unlike other glycans with high Gal/GalNAc-lectin affinity such as lactose or Gal (Ravdin et al. 1981, Cano-Mancera et al. 1987), HMO are only minimally digested and absorbed in the small intestine and reach the colon as the actual site of *E. histolytica* infection. If confirmed *in vivo*, the antiadhesive antimicrobial effects of HMO may provide one explanation for why breast-fed infants are at lower risk to acquire *E. histolytica* infections than formula-fed infants (Islam et al. 1988).

Antiadhesive antimicrobial effects may not only be relevant to enteric infections. Human milk often covers the mucosal surfaces in the infant's nasopharyngeal regions and occasionally reaches the upper respiratory tract during episodes of aspiration. Breast-fed infants are less likely to develop otitis media caused by *Streptococcus pneumonia*, *Pseudomonas aeruginosa* or *Haemophilus influenzae* and are also at lower risk to develop respiratory syncytial virus (RSV) (Downham et al. 1976, Abrahams et al. 2011). These microbial pathogens employ lectin-glycan interactions to initiate infection, and HMO have been shown to block their attachment - at least *in vitro* (Andersson et al. 1986, Devaraj et al. 1994, Lesman-Movshovich et al. 2003, Malhotra et al. 2003). Similarly, HMO are absorbed and excreted with the urine, and they reduce uropathogenic *E. coli*-induced hemagglutination (Martin-Sosa et al. 2002), suggesting that HMO also reduce urinary tract infections. In conclusion, the antiadhesive antimicrobial effects of HMO may contribute to the lower incidence of intestinal, upper respiratory and urinary tract infections in breast-fed compared to formula-fed infants (*Figure 5B*).

**Modulators of Intestinal Epithelial Cell Responses**

HMO interfere with host-microbial interactions by serving as prebiotics or antiadhesive antimicrobials, but may also directly modulate host intestinal epithelial cell responses. Incubating cultured human intestinal epithelial cell lines with the HMO 3′-sialyllactose lowers the gene expression of sialyltransferases ST3Gal1, ST3Gal2, and ST3Gal4 and diminishes α2-3-
and α2-6-sialylation on cell surface glycans (Angeloni et al. 2005). As a consequence, binding of enteropathogenic *E. coli* (EPEC) is significantly reduced as it uses sialylated cell surface glycans to attach to the host’s intestinal epithelial cell. It needs to be further investigated how these results translate to *in vivo* and whether they are relevant to changing the course of infectious diseases in the neonate. This was however the first study to show that HMO are able to directly affect intestinal epithelial cells, induce differential gene expression and modulate a cell response.

In the meantime, other studies have confirmed that HMO directly modulate intestinal epithelial cell responses. HMO reduce cell growth and induce differentiation and apoptosis in cultured human intestinal epithelial cells by altering growth-related cell cycle genes (Kuntz et al. 2008, Kuntz et al. 2009).

The combined observations from these *in vitro* studies strongly suggest that HMO can directly interact with the infant’s intestinal epithelial cells, affect gene expression and reprogram the cell cycle as well as cell surface glycosylation (*Figure 5C*). It requires further investigation to determine which receptors and signaling pathways HMO employ to trigger differential gene expression. Epidermal growth factor receptor (EGFR) and Ras/Raf/ERK signaling may be involved (Kuntz et al. 2009), but whether HMO directly interact with EGFR or indirectly modulate its signal remains to be determined. Also, whether these *in vitro* observations are viable in animal models and translate to the human neonate remains to be investigated.

**Immune modulators**

While HMO-mediated changes in the infant’s microbiota composition or intestinal epithelial cell response may indirectly affect the infant’s immune system, results from *in vitro* studies suggest that HMO also directly modulate immune responses. HMO may either act locally on cells of the
mucosa-associated lymphoid tissues (MALT) or on a systemic level since approximately 1% of the HMO are absorbed and reach the systemic circulation (Rudloff et al. 1996, Gnoth et al. 2001, Rudloff et al. 2011). The number of interferon-γ-producing CD3+CD4+ and CD3+CD8+ lymphocytes as well as interleukin-13 (IL-13)-producing CD3+CD8+ lymphocytes increases when cord blood T-cells are exposed to sialylated HMO (Eiwegger et al. 2004). This observation led the study authors to speculate that sialylated HMO influence lymphocyte maturation and promote a shift in T-cell response towards a more balanced Th1/Th2-cytokine production and low-level immunity (Figure 5D). Sialylated HMO also reduce IL-4 production in a subset of lymphocytes from adult patients with peanut-allergy, which led to the conclusion that certain sialylated HMO may contribute to allergy prevention (Eiwegger et al. 2010).

LNFP III (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc) and LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), two HMO whose structures are also found on helminth parasite glycans, expand peritoneal macrophage populations capable of suppressing naïve CD4+ T-cell responses (Atochina et al. 2001, Terrazas et al. 2001). In addition, LNFP III stimulates macrophage activity in vitro and increases secretion of prostaglandin E2, IL-10, and TNFα (Atochina et al. 2005). The physiological relevance of these in vitro observations remains to be elucidated.

It is currently unknown which receptors and signaling pathways transduce HMO-mediated effects on lymphocyte cytokine production or macrophage stimulation. Several different lectins are involved in the immune system, and their glycan-binding specificity suggests that HMO may interfere with some of these processes. Siglecs (sialic acid binding Ig-like lectins) for example are cell surface receptors and members of the immunoglobulin superfamily that recognize sialic acids. Siglecs are involved in the immune system in multiple ways (Crocker et al. 2007) and have been shown to bind sialylated HMO (Koliwer-Brandl 2011). Galectins are β-galactoside-
binding lectins that modulate immune responses (Rabinovich et al. 2002). HMO are β-galactosides that often contain β1-3- or β1-4-linked Gal at their non-reducing end and could potentially target galectin-mediated interactions. Whether HMO modulate siglec- or galectin-mediated immune responses and impact infant physiology needs further investigation. Some in vitro and ex vivo data however suggest that HMO interfere with another family of lectins, the selectins.

Selectins are C-type lectins and cell adhesion molecules that mediate the earliest stages of leukocyte trafficking and platelet-neutrophil complex (PNC) formation. At sites of inflammation, leukocytes need to migrate from the blood stream through the endothelium into subendothelial regions of inflammation (Osborn 1990, Springer 1994). Induced by proinflammatory cytokines, endothelial cells express P- and E-selectin, which bind to glycoconjugates on leukocytes passing by with the blood stream. This initial contact decelerates the leukocytes and makes them roll over the endothelial cell layer. Subsequently, additional adhesion molecules bring leukocytes to a complete stop and facilitate their transmigration into subendothelial regions. Initial selectin-mediated rolling is essential for leukocyte extravasation and mucosal infiltration.

Selectins also initiate PNC formation (Cerletti et al. 1999, Evangelista et al. 1999, Peters et al. 1999). Activated platelets express P-selectin that binds to glycoconjugate ligands on neutrophil surfaces and cause the neutrophils to increase their expression of adhesion molecules, capacity for phagocytosis and production of reactive oxygen species. Again, selectin-mediated platelet-neutrophil complex formation is essential to activate this highly reactive neutrophil subpopulation.

Selectins bind to glycans that carry sialylated Lewis blood group epitopes (Varki 1997), which are sialylated and fucosylated lacto-N-biose (Galβ1-3GlcNAc) or N-acetyllactosamines (Galβ1-
4GlcNAc) – very similar to HMO. In fact, HMO contain Lewis blood group antigens (Rudloff et al. 2002) and are able to reduce selectin-mediated cell-cell interactions (Bode et al. 2004a, Bode et al. 2004b) (Figure 5E). Sialylated HMO reduce leukocyte rolling and adhesion in an in vitro flow model with TNFα-activated human endothelial cells (Bode et al. 2004a). Similarly, sialylated HMO reduce PNC formation and subsequent neutrophil activation in an ex vivo model with whole human blood (Bode et al. 2004b). In both cases, non-sialylated HMO are ineffective and pooled HMO are more effective than monovalent sialyl-Lewis X, indicating the importance of sialic acid and suggesting potential multivalent interactions with higher molecular HMO that carry more than one sialylated blood group epitope. Whether these in vitro and ex vivo observations translate to health benefits for the breast-fed neonate remains to be elucidated.

Increased mucosal neutrophil infiltration and activation occurs in early stages of diseases like necrotizing enterocolitis (Stefanutti et al. 2005), but whether HMO can reduce these potentially detrimental immune responses and protect neonates from disease is currently unknown.

Natural Protection against Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is one of the most common and often fatal disorders in preterm infants (Uauy et al. 1991, Holman et al. 1997, Neu et al. 2011). Between 5 and 10% of very-low-birth weight infants (<1,500g) develop NEC (Holman et al. 2006). More than a quarter of them die, and the survivors are often faced with severe neurological complications (Holman et al. 1997, Rees et al. 2007). NEC etiology and pathogenesis are poorly understood. There are currently no biomarkers to identify neonates at risk to develop NEC. Treatment is limited and surgical resection of the necrotic intestine often the last remaining option that comes with long-term complications associated with short bowel syndrome. Most intriguingly, breast-fed infants are at a 6- to 10-fold lower risk to develop NEC than formula-fed infants (Lucas et al. 1990a, Schanler et al. 2005, Sisk et al. 2007). The significant differences in HMO amount and composition between human milk and infant formula led to the hypothesis that HMO contribute
to the protective effects of breast-feeding against NEC. Human intervention studies to test this hypothesis are unfeasible due to the lack of available HMO required for a well-powered and well-controlled design. Alternatively, studies in an established rat model of the disease show that HMO indeed protect from NEC (Jantscher-Krenn et al. 2011). Survival rates and pathology scores significantly improve when HMO are added to the orally gavaged formula. A single HMO, disialyllacto-N-tetraose (DSLNT, Figure 2E), seems responsible for the protection, which suggests a highly structure-specific and potentially host receptor-mediated effect. It remains to be investigated how DSLNT protects from NEC and whether the results translate from the animal model to human neonates.

**Nutrients for Brain development**

Breast-fed preterm infants have superior developmental scores at 18 months of age and higher intelligence quotients at the age of 7 (Lucas et al. 1990b, Lucas et al. 1992). A body of evidence suggests that brain development and cognition in part depend on sialic acid-containing gangliosides and poly-sialic acid containing glycoproteins (reviewed in (Wang 2009). Sialic acid concentrations in the brain more than double between a few months prior to birth and a couple of years after birth (Svennerholm et al. 1989). Animal studies suggest that dietary sialic acid is an essential nutrient to serve the high sialic acid demand during pre- and postnatal stages of brain development (Carlson et al. 1986, Wang et al. 2007). For example, learning and memory increases in piglets when the sow milk replacer is supplemented with sialylated casein glycomacropeptide (Wang et al. 2007). Human milk is a rich source of sialic acid (Wang et al. 2001), and post-mortem analysis on human neonates showed that ganglioside- and protein-bound sialic acid concentrations are significantly higher in the brains of breast-fed infants compared to infants fed with formula that contained lower amounts of sialic acid than human milk (Wang et al. 2003). Sialylated HMO contribute to the majority of sialic acid in human milk. The amount of sialic acid from HMO is two- to three fold higher than that from glycoproteins,
and sialic acid in glycolipids accounts for only about 1% of the total sialic acid in human milk (Wang et al. 2001). It remains to be investigated whether sialylated HMO are the primary sialic acid carrier that provides the developing brain with this seemingly essential nutrient and contribute to superior developmental scores and intelligence quotients in breast-fed infants (Figure 5F).

**Effects on the Mother**

So far, all of the potential benefits of HMO relate to the breast-fed infant. It is however possible that HMO also affect the breast-feeding mother and the underlying mechanisms may be similar. Human milk *per se* is not sterile and contains complex bacterial communities that are often highly personalized to the lactating woman (Martin et al. 2007, Hunt et al. 2011). These bacteria can be regarded as natural probiotics to inoculate the infant’s intestinal microbiota, but they could also be regarded as potential commensals that modulate the mother’s milk composition or pathogens that cause diseases like mastitis. HMO may influence the bacterial communities in milk in the mammary gland by serving as prebiotics or antiadhesive antimicrobials or by directly modulating mammary gland epithelial cell responses or local immune responses, very similar to what has been described in the context of the neonate. *Staphylococcus*, for example, is a major cause of mastitis, defined as inflammation of the breast that is associated with redness, swelling, painful lactation, and sometimes fever or flu-like symptoms (Barbosa-Cesnik et al. 2003, Delgado et al. 2009). Some *Staphylococcus* strains bind to 2’-fucosyllactose in a biosensor-based assay (Lane et al. 2011), but whether 2’-fucosyllactose or other HMO interact with *Staphylococcus* strains to reduce or increase the risk of mastitis is unknown. In addition, HMO appear in pregnant women’s urine shortly prior to parturition (Date 1964, Hallgren et al. 1977a, Hallgren et al. 1977c, Hallgren et al. 1977b). These observations indicate retrograde “leakage” into the circulation and suggest potential systemic effects not only in the breast-fed infant, but also in the breast-feeding mother.
Oligosaccharides in the Milk of Other Mammals

Oligosaccharides in the milk of many other mammals have been studied over the years, but no other animal matches the high amount and high structural diversity of human milk oligosaccharides (reviewed in (Urashima et al. 2001). Two different groups recently analyzed oligosaccharides in the milk of New and Old World monkeys and apes (Goto et al. 2010, Tao et al. 2011). In general, the oligosaccharides in primate milk, including humans, are more complex and exhibit greater diversity compared to those in non-primate milk. In humans 50-80% of the oligosaccharides are fucosylated depending on the Se/Le group, which is followed by chimpanzees at around 50% and gorillas with only 15%. Most other species show very low levels of fucosylation (<1%). In humans, 10-30% of the oligosaccharides are sialylated and similar values are found in chimpanzees, rhesus and gorillas. Interestingly, primate milk oligosaccharide cluster analysis does not follow primate phylogeny, suggesting an independent emergence of milk oligosaccharides, potentially driven by distinct pathogen exposures (Tao et al. 2011).

Oligosaccharide concentrations in milk of most farm animals including cows, goats, sheep and pigs are 100-1,000-fold lower than that in human milk, with a lower number of different oligosaccharides, a higher abundance of sialylated and a lower abundance of fucosylated oligosaccharides (Saito et al. 1981, Saito et al. 1984, Urashima et al. 1991, Martinez-Ferez 2006, Nakajima et al. 2006, Tao et al. 2010). Table I highlights significant differences in oligosaccharide concentration and composition between human milk and bovine milk, which forms the basis for most infant formula. In addition, bovine milk oligosaccharides contain not only Neu5Ac, but also some of the non-human Sia derivative N-glycolylneuraminic acid (Neu5Gc). It remains to be investigated whether incorporation of infant formula-derived exogenous Neu5Gc into rapidly growing neonatal tissues affect infant health (Irie et al. 1998,
Tangvoranuntakul et al. 2003, Taylor et al. 2010). In addition, bovine milk also contains α3′-galactosyllactose (Galα1-3Galβ1-4Glc) that is not found in human milk (Urashima et al. 1991). It is unknown whether the concentrations of non-human αGal-containing oligosaccharides in infant formula are sufficient to trigger adverse IgE-mediated responses (Chung et al. 2008, Commins et al. 2009).

**Oligosaccharides in Infant Formula**

Oligosaccharides in the milk of farm animals are much less abundant and structurally less complex than oligosaccharides in human milk, and no other natural resources are available to provide access to large amounts of HMO. Hence, infant formula does not provide the human neonate with HMO. As an alternative and in an attempt to mimic the multiple benefits of HMO, other, non-human milk oligosaccharides are currently added to infant formula, including galactooligosaccharides (GOS) and fructooligosaccharides (FOS).

GOS are galactose oligomers with a degree of polymerization (DP) between 3 and 10 (mostly 3, 4 and 5) that are synthesized from lactose by enzymatic transgalactosylation using β-galactosidases from yeast or bacteria and lactose as the substrate (Fransen et al. 1998). Depending on enzyme source, GOS contain β1-4 and β1-6, but also β1-2 or β1-3 linkages, leading to a variety of different structural isomers (Coulier et al. 2009).

FOS are mostly β2-1-linked fructose oligomers of the inulin-type often extracted from Compositae family plants like chicory (Roberfroid 2005, Roberfroid 2007b). The DP of chicory inulin varies between 2 and more than 60, and the polymers often carry glucose at the reducing end. FOS is produced from inulin using an endoinulinase that cleaves the polymers into smaller oligomers with or without glucose at the reducing end (Cho et al. 2001, Park et al. 2001). FOS
can also be synthesized by transfructosylation using β-fructosidases from yeast or bacteria and sucrose as the substrate (Lafraya et al. 2011, Tian et al. 2011). These synthetic FOS usually carry glucose at the reducing end and their DP is often less than 5.

It is important to note that galactose and fructose oligomers do not naturally occur in human milk. In fact, the fructose monomer itself is not found in human milk. On the other hand, GOS and FOS are neither fucosylated nor sialylated. Despite their structural differences compared to HMO, ingestion of GOS and FOS influences the microbiota composition in the infant’s feces and provides other benefits that are extensively reviewed elsewhere (Seifert et al. 2007, Boehm et al. 2008, Macfarlane et al. 2008, Rijnierse et al. 2011). A defined mixture of GOS and FOS reduces the incidence of atopic dermatitis during the first six months of life (Moro et al. 2006) and subsequent allergic manifestations and infections during the first two years of life (Arslanoglu et al. 2008). Long-term health benefits and risks of providing infants with significant amounts of these non-human milk glycans need to be further investigated.

GOS and FOS are non-sialylated, but the carboxyl-group of sialic acid introduces a negative charge critical to some HMO effects. In an attempt to more closely resemble the composition of oligosaccharides naturally occurring in human milk, a pectin hydrolysate consistent of galacturonic acid oligomers has recently been studied as an additional infant formula oligosaccharide (Westerbeek et al. 2011a, Westerbeek et al. 2011b, Westerbeek et al. 2011c). While galacturonic acid provides a negatively charged carboxyl-group, its overall structure is very different from sialic acid. Hence, it is not surprising that pectin-derived acidic oligosaccharides (pAOS) so far failed to affect infant stool viscosity, frequency, pH or feeding tolerance (Westerbeek et al. 2011a). Additional studies are required to assess short- and long term benefits or adverse effects of introducing non-human milk galacturonic acid oligomers in early infant feeding.
Current Roadblocks and Future Opportunities

Future research on HMO will likely continue to elucidate and verify the beneficial effects of HMO for the breast-fed infant, but it will be intriguing to observe whether and how HMO impact the health of the breast-feeding mother and the composition of other milk components. One of the biggest roadblocks in HMO research remains to be the limited availability of HMO resources needed to better understand the underlying mechanisms of action and to confirm that the observed effects translate to measurable health benefits for the neonate. Due to advances in chemical and enzymatic carbohydrate synthesis, HMO tri- and tetrasaccharides have recently become available in kg quantities, which is going to boost preclinical research and enable first clinical intervention studies. Still, HMO remain expensive, and government agencies as well as formula companies are faced with making decisions on which of the available HMO to test and what primary outcome to study. Is there sufficient preclinical data to warrant a multi million-dollar intervention study to assesses whether 2'-fucosyllactose or other HMO reduce episodes of infectious diarrhea? Is there enough data to support potentially even more expensive studies to determine whether 3'- or 6'-sialyllactose improve infant learning and cognition?

At this point, only a handful of HMO tri- and tetrasaccharides are available in sufficient quantities to move forward, but what if a mix of structurally more complex HMO is needed to confer significant health benefits? In the end, the human mammary gland produces not only one or two HMO, but more than one hundred different oligosaccharides. Insights into how HMO are synthesized in the human mammary gland may help develop novel strategies and technologies to generate complex mixtures of HMO.

HMO research has come a long way since 1900 when Moro and Tissier first observed that human milk feeding affects infant intestinal microbiota composition (Moro 1900, Tissier 1900).
Many questions about HMO biosynthesis, metabolism and health benefits however remain unanswered and create exciting opportunities for future generations of dedicated scientists. History shows that it was the collaboration between György and Kuhn, two scientists with entirely different backgrounds, that eventually led to the discovery of HMO (Gauhe et al. 1954, György 1954, György et al. 1954a, György et al. 1954b, Rose et al. 1954). It will likely be these multi-discipline collaborations that involve pediatricians, nutritional scientists, microbiologists, chemists, glycobiologists and many others, that will continue to create the biggest impact on HMO research.
FUNDING

The author is supported by a Pathway to Independence Award of the National Institutes of Health [DK78668].

ACKNOWLEDGEMENTS

I express my deepest gratitude to my PhD advisors Clemens Kunz and Silvia Rudloff at the Justus-Liebig-University in Giessen, Germany, who initiated my excitement for HMO research, to my postdoctoral advisor Hudson Freeze at the Burnham Institute in La Jolla, California, who taught me the essentials of glycobiology, and to Ajit Varki and Jeff Esko at the University of California, San Diego, for their continuous inspiration and support. I thank the dedicated and hard working members of my lab at the University of California, San Diego, better known as the “Milk Gang”.

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ABBREVIATIONS

2'FL 2'-fucosyllactose
DC Dendritic Cell-Specific ICAM3-Grabbing Non-integrin
DP degree of polymerization
DSLNT Disialyllacto-N-tetraose
EGFR Epidermal growth factor receptor
EPEC enteropathogenic *E. coli*
Fuc Fucose
FUT fucosyltransferase
Gal Galactose
GalT Galactosyltransferase
Glc Glucose
GlcNAc N-acetylgalcosamine
HIV human immunodeficiency virus
HMO Human Milk Oligosaccharides
IL interleukin
Le Lewis
LNFP Lacto-N-fucopentaose
LNnT Lacto-N-neotetraose
LNT Lacto-N-tetraose
LST Sialyllacto-N-tetraose
MALT mucosa-associated lymphoid tissue
NEC Necrotizing Enterocolitis
Neu5Ac N-acetylneuraminic acid
Neu5Gc N-glycolylneuraminic acid
<table>
<thead>
<tr>
<th>pAOS</th>
<th>pectin-derived acidic oligosaccharides</th>
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<tr>
<td>PNC</td>
<td>platelet-neutrophil complex</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>Se</td>
<td>Secretor</td>
</tr>
<tr>
<td>Sia</td>
<td>Sialic acid</td>
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<tr>
<td>STGal</td>
<td>(\beta)-galactoside sialyltransferase</td>
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REFERENCES


LEGENDS TO FIGURES

Figure 1. Pioneers in HMO research in the 20th century

HMO research originates at the end of the 19th century with parallel work by pediatricians and microbiologists that studied the health benefits of human milk (top half of the time scale) and chemists that characterized the carbohydrates abundant in human milk (bottom half of the time scale). Towards the middle of the 20th century scientists from both disciplines closely collaborated, which led to the discovery that human milk “gynolactose” is the “bifidus factor” and consists of oligosaccharides. Since then more than a hundred different HMO have been isolated and characterized and accumulating data suggests that HMO benefit the breast-fed neonate in multiple ways. (Black arrows indicate direct mentor-mentee relationships over the course of the century).

Figure 2. HMO Blueprint and selected HMO structures

(A) HMO follow a basic structural blueprint. (Monosaccharide key is shown at the bottom of the figure.) (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-N-biose (type I) or N-acetyllactosamine (type II) disaccharides. Addition of disaccharides to each other in β1-3 linkage leads to linear chain elongation (para-HMO); a β1-6 linkage between two disaccharides introduces chain branching (iso-HMO). (D) Elongated type I or type II chains can be fucosylated in different linkages to form a variety of structural isomers, some of which have Lewis blood group specificity (Figure 3). (E) The elongated chains can also be sialylated in different linkages to form structural isomers. Disialylated Lacto-N-tetraose (bottom right) prevents necrotizing enterocolitis in neonatal rats.
Figure 3. Secretor- and Lewis-dependent HMO fucosylation

HMO fucosylation highly depends on a woman’s Secretor and Lewis blood group status, and allows for the distinction of four milk groups. Fucosyltransferase FUT2 is encoded by the Secretor gene (Se) and facilitates the addition of Fuc to terminal Gal in $\alpha 1-2$ linkage. Fucosyltransferase FUT3 is encoded by the Lewis gene (Le) and catalyzes the addition of Fuc to subterminal GlcNAc on type I chains in $\alpha 1-4$ linkage. If both FUT2 and FUT3 are expressed, milk contains HMO with Lewis b antigens (highlighted). If only FUT3 is expressed, milk contains HMO with Lewis a antigens (highlighted). If FUT3 is not expressed, HMO contain neither Lewis a nor Lewis b antigens.

Figure 4. Postulated HMO Biosynthesis

While lactose synthesis in the Golgi of mammary gland epithelial cells has been well described and is catalyzed by lactose synthase (LS), subsequent HMO biosynthesis remains poorly understood. Speculations on enzymes (shaded background) involved in HMO biosynthesis are based on other known glycan synthesis pathways, but data to prove their involvement in HMO biosynthesis is often missing, which raises many questions that are pointed out in the figure with question marks and are further explained in the text. Enzymes known to be involved are highlighted in bold. Enzymes that are speculated to be involved are italicized.

Figure 5. Postulated HMO Effects

HMO may benefit the breast-fed infant in multiple different ways. (A) HMO are prebiotics that serve as metabolic substrates for beneficial bacteria (green) and provides them with a growth advantage over potential pathogens (purple). (B) HMO are antiadhesive antimicrobials that serve as soluble glycan receptor decoys and prevent pathogen attachment. (C) HMO directly affect intestinal epithelial cells and modulate their gene expression, which leads to changes in
cell surface glycans and other cell responses. (D) HMO modulate lymphocyte cytokine production, potentially leading to a more balanced Th1/Th2 response. (E) HMO reduce selectin-mediated cell-cell interactions in the immune system and decrease leukocyte rolling on activated endothelial cells, potentially leading to reduced mucosal leukocyte infiltration and activation. (F) HMO provide sialic acid as potentially essential nutrients for brain development and cognition. (Center photo taken from author’s personal collection)
### TABLES

Table I. Macronutrients and HMO in mature human and bovine milk (approximate values)

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</table>

<sup>#</sup> depending on the woman’s Secretor/Lewis blood group status

<sup>1</sup> Data from (Hale 2007)

<sup>2</sup> Data complied from the following references (Coppa et al. 1999, Kunz et al. 1999, Newburg et al. 2000, Chaturvedi et al. 2001a, Davidson et al. 2004, Bao et al. 2007, Gabrielli et al. 2011)

<sup>3</sup> Data from (Gopal et al. 2000)

<sup>4</sup> Data complied from the following references (Ninonuevo et al. 2006, Wu et al. 2010, Wu et al. 2011) and reviewed in (Kobata 2010)

<sup>5</sup> Data from (Tao et al. 2008, Tao et al. 2009)
Fig. 1

- Intestinal bacteria
- Impact on infant physiology
- Different in breast- vs bottle-fed milk
- ’Bifidus-factor’ in human milk
- Elucidation of nutritional and biological benefits of HMO (prebiotic, anti-adhesive, etc.)
- Escherich 1886
- Mero 1900
- Schönfeld 1926
- Eschbach
- Doniger
- Carbohydrates different in human vs bovine milk
- Lespagnolet
- Polonowski
- Additional carbohydrate fraction in human milk
- ’Gynolactose’
- György
- György 1054
- Kuhn
- Isolation and characterization of 100+ different oligosaccharides
Fig. 2
**Group 1:** Lewis-positive Secretor (Se+Le+)
Lewis a-b+

![Diagram for Group 1: Lewis-positive Secretor (Se+Le+)](image1)

**Group 2:** Lewis-positive Nonsecretor (Se-Le+)
Lewis a+b-

![Diagram for Group 2: Lewis-positive Nonsecretor (Se-Le+)](image2)

**Group 3:** Lewis-negative Secretor (Se+Le-)
Lewis a-b-

![Diagram for Group 3: Lewis-negative Secretor (Se+Le-)](image3)

**Group 4:** Lewis-negative Nonsecretor (Se-Le-)
Lewis a-b-

![Diagram for Group 4: Lewis-negative Nonsecretor (Se-Le-)](image4)

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Fig. 3
Fig. 4
Fig. 5