Human Milk Oligosaccharides: Factors Affecting Their Composition and Their Physiological Significance

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Abstract

Human milk oligosaccharides (HMOs) are elongations of the milk sugar lactose by galactose, N-acetylglucosamine, fucose; and sialic acid. The HMO composition of breast milk is strongly influenced by polymorphisms of the maternal fucosyltransferases, FUT2 and FUT3, and by the stage of lactation. Clinical observational studies with breastfed infant-mother dyads associate specific HMOs with infant gut microbiota, morbidity, infectious diarrhea, and allergies. Observational and basic research data suggest that HMOs influence the establishment of early-life microbiota and mucosal immunity and inhibit pathogens, thereby contributing to protection from infections. Clinical intervention trials with infant formula supplemented with the single HMO, 2′-fucosyllactose (2′FL), or with 2 HMOs, 2′FL and lacto-N-neotetraose (LNnT), demonstrated that they allow for age-appropriate growth and are well tolerated. A priori defined exploratory outcomes related feeding an infant formula with 2 HMOs to fewer reported illnesses of the lower respiratory tract and reduced need for antibiotics during the first year of life compared to feeding a control formula. In parallel, early-life microbiota composition shifted towards that of breastfed infants. Together, HMOs likely contribute to immune protection in part through their effect on early-life gut microbiota, findings that warrant further clinical research to improve our understanding of HMO biology and significance for infant nutrition.

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Introduction

What are human milk oligosaccharides (HMOs)? What is their importance for infant nutrition? These questions have intrigued scientists and pediatricians alike for over a century. Advances in analytics as well as large-scale synthesis...
technologies stimulated great progress in recent years. These provided the materials and tools that enabled the detailed and accurate measurement of HMO quality and quantity, and the study of HMOs in basic research models, and through clinical observational studies and intervention trials.

“HMOs are not HMOs,” meaning that one specific HMO is not equal to another HMO, especially when considering structure-function relationships. Chemically, HMOs are elongations of the milk-specific sugar lactose in different linkages by one or a combination of the following monosaccharides: L-fucose (Fuc), D-galactose (Gal), N-acetyl-D-glucosamine (GlcNAc), and N-acetylneuraminic acid (sialic acid). Gal and GlcNAc generally elongate lactose as a disaccharide Gal-GlcNAc. The numerous and diverse HMOs produced might be categorized by specific structural features brought about by different glycosyltransferases involved in their synthesis. However, many HMOs combine different structural features.

Breast milk, the recommended and naturally adapted nutrition for infants, is associated with a reduced risk for infection-related illnesses and possibly for diabetes and overweight, while the situation for allergies is less clear [1]. This suggests that breast-milk-specific components such as HMOs and other bioactives may contribute to such benefits.

Due to their structural similarity with mucosal glycans and their nondigestible nature, HMOs expectedly affect numerous glycan-mediated processes like the colonization of the early-life microbiota and the infectivity of pathogens (Fig. 1a). Based on clinical observational and basic research data, HMOs act in a structure-function-specific way helping the (i) establishment of a mucous-adapted microbiome, (ii) resistance to pathogens, and (iii) reactivity of the mucosal barrier and immunity, thereby contributing to immune protection.

Here, we briefly review genetic and environmental factors affecting HMO composition in breast milk and the physiological role of HMOs as supported by clinical observation studies, preclinical research on mode of action, and insights from clinical intervention trials.

Maternal Glycosyltransferase Polymorphisms Affect HMO Composition

HMOs resemble the blood group antigens and further sialylated glycans that cover the human mucosa. The same glycosyltransferases are generally involved in the synthesis of mucosal cell glycans and mammary gland-expressed HMOs. The fucosyltransferases FUT2 (secretor gene) and FUT3 (Lewis gene) are the best described due to their natural polymorphisms in humans [2] (Fig. 2a). Spe-
specific genetic polymorphisms abolish their respective enzyme activity. Thus, specific HMO structures that depend on FUT2 or FUT3 can be identified. FUT2-dependent HMOs all contain α1,2-linked fucose, for example 2′-fucosyllactose (2′FL), lactodifucosyllactose (LDFT), and lacto-N-fucosylpentose (LNFP)-I. Interestingly, trace amounts of 2′FL were found in breast milk of presumed FUT2-
Fig. 2. Human milk oligosaccharide (HMO) composition 3 months postpartum by FUT2 and FUT3 status with schematic illustration of typical HMO in one or the other group (a). HMO dynamics at different stages of lactation (replotted from Austin et al. [3]) depicting mean concentrations with standard deviations (b).
negative mothers in Asian populations [3, 4], indicating that the nature of these inactivating polymorphisms and thus the HMO profile may be population specific [5]. Typical FUT3-dependent HMOs are LNFP-II, lacto-N-difucosylhexose (LNDFH)-I containing α\textsubscript{1,4}-linked fucose, and to a lesser extent 3-fucosyllactose (3FL) and LDFT containing α\textsubscript{1,3}-linked fucose. In breast milk that does not contain any detectable LNFP-II, reduced amounts of HMOs with α\textsubscript{1,3}-linked fucose on glucose and increased amounts of those with an α\textsubscript{1,3}-linked Fuc on GlcNAc are found. Hence, another FUT (e.g., FUT4, FUT5, FUT6, FUT7, or FUT9) is also involved in HMO formation with an α\textsubscript{1,3}-linked Fuc on GlcNAc and glucose.

The absence of a functional FUT2 or FUT2 and FUT3 affects the concentration of total HMOs in milk when expressed as the sum of all quantified HMOs [2] (Fig. 2). While some HMOs increase when FUT2 is missing (e.g., LNnT and 3FL), in the absence of fucosylation additional larger nonfucosylated HMOs might also be produced.

To date, no common genetic polymorphisms for sialylated HMOs have been described, indicating that if inactivating polymorphisms in sialyltransferase genes exist, they are extremely rare. From mouse studies, the sialyltransferases ST6Gal1 and ST3Gal4 are involved in the synthesis of 6′-sialyllactose (6′SL) and 3′-sialyllactose (3′SL), respectively, with a further sialyltransferase, probably ST3Gal1, also making 3′SL [6].

Another mechanism affecting HMO composition is probably the donor and acceptor substrate availability, as suggested by the increase in 3FL when the major fucosyl-HMO 2′FL decreases in concentration [3].

Interestingly, HMO concentrations change during the stage of lactation with different HMOs showing different dynamics [3]. HMOs like 6′SL or LNnT decrease more rapidly during the first weeks of lactation, while 2′FL and 3′SL, for example, decrease more slowly over a longer time period, and again others, like 3FL, actually increase in concentration with time of lactation (Fig. 2b).

Such compositional changes due to the genetic background of mothers and stage of lactation can confound observations relating HMOs to clinical parameters in the breastfed infants and, therefore, need to be considered.

**HMO Composition and Maternal Diet, Gestational Age, and Physiological State of the Infant**

HMO concentrations in colostrum, transitional milk, and mature milk seem not to change between mothers giving birth to preterm (n = 18; gestational age <37 weeks) and term (n = 14; gestational age ≥37 weeks) infants [2]. Further,
fucosylated and sialylated HMOs were reported to be similar between preterm and term milk, although preterm milk seemed more variable in the expression of fucosylated HMOs [7].

Today, we do not know whether and how maternal diet might influence HMO composition. A recent observational study including 33 breastfeeding mothers and their infants from the Gambia, Africa, reported a significantly higher HMO content in milk at 20 weeks of lactation in the dry season \( (n = 21) \) than the wet season \( (n = 12) \) [8]. The authors propose a possible link to the higher energy intake during the dry season. In 2 other African mother-infant cohorts from Malawi (\( n = 88 \) and \( n = 215 \)), total HMOs and also sialyl- and fucosyl-HMOs were lower 6 months postpartum in the breast milk of mothers having severely stunted infants compared to those with normal-size infants [9]. These studies suggest that maternal nutritional and health status may affect HMO composition.

By analogy, higher maternal body mass index and gestational weight gain, which generally reflects an altered metabolic physiology, might affect HMO composition. Studies to this end are currently ongoing [10; Binia et al.: abstract at FASEB Science Research Conferences in 2017]. Suitable studies are warranted to investigate possible alterations in HMO composition due to maternal energy and specific nutrient intake.

**The HMO Composition Is Associated with the Gut Microbiota in Infants**

The early-life microbiome has a major impact on the developing immune system, itself being an important element by providing pathogen colonization resistance, for example. Interestingly, the establishing intestinal microbiota also contributes, via an innate lymphoid cell-mediated process, to improved protection against respiratory tract infection [11]. The pioneers of human milk and breastfeeding research observed a strong link between breastfeeding and immune protection to infectious morbidity and mortality. Breastfed infants were recognized to harbor an early gut microbiota dominated by bifidobacteria, not seen in formula-fed infants, and a human-milk-specific “bifidofactor” was identified in the HMO fraction of breast milk [12].

From research on early-life microbiota, we know that bifidobacteria can utilize and grow on different individual HMOs in a strain-specific way [13, 14]. Several studies observed an increased bacterial metabolic activity upon growth on HMOs, exemplified by the formation of the short-chain fatty acid acetate [15, 16]. Noteworthy, numerous potentially pathogenic bacteria from the Enterobacteriaceae group were shown not to grow on individual HMOs as the sole carbon source [17], while growth of other pathogens, like *Streptococ-
*c*us agalactiae (group B *Streptococcus*, GBS) was shown to be inhibited by HMOs [18, 19].

Recently, LNNt in breast milk was associated with *Bifidobacterium longum* ssp. *infantis* abundance [8]. In bi-associated gnotobiotic mice harboring only 1 *Bacteroides* and 1 *B. longum* ssp. *infantis* strain, LNNt lead to bifidobacteria dominance although both bacteria could actually use LNNt in vitro [20]. In gnotobiotic mice humanized with 7 human microbes, *B. longum* ssp. *infantis* also showed higher abundance when these mice were fed 2′FL combined with LNNt as compared to LNNt alone [Sprenger et al., unpubl. observation], although *B. longum* ssp. *infantis* is able to grow on many different HMOs, including LNNt, as substrate [13].

Genomic and glycomic analyses in infants provided further evidence for a role of HMOs in shaping the early infant gut microbiome, revealing associations between individual HMOs and bacterial genera in infant stool [21–23]. A *Bifidobacterium*-dominated gut microbiota in breastfed infants (*n* = 105) at 4 months of age was associated with breast milk containing FUT2-HMOs [24]. The FUT2 status of the infants and its possible confounding effects on the infant microbiota profile were not assessed, despite earlier data proposing the FUT2 status itself can influence the gut microbiota at least in adults [25]. In another cohort, the analysis of a relatively small subgroup of infants exclusively breastfed for 4 months (*n* = 14) showed an association of maternal FUT2-positive status with higher *Bifidobacterium* abundance up to 2–3 years of age [26]. However, no statistically significant HMO effects on global *Bifidobacterium* shifts were reported in another recent study of 33 Gambian mothers and infants [8], while the abundance of individual bifidobacteria like *B. longum* ssp. *infantis* still correlated with LNNt concentrations in breast milk. These first reports reveal the need for larger observational studies of similar design, including comprehensive HMO analysis of breast milk and infant FUT2 phenotyping to gain a more robust understanding of the link between HMO and infant gut microbiome composition.

Today, clinical observations in conjunction with basic research data suggest that FUT2-HMOs, like 2′FL and LNFP-I, but likely also other non-FUT2-dependent HMOs, like LNNt for example, are involved in the establishment of a *Bifidobacterium*-dominated early-life gut microbiota. In vitro studies help to understand HMO-related microbial metabolic capacities and strain specificities, while animal and human observational studies indicate that the interaction between bacteria and the gut mucosa reflect a more complex picture. Hence, with infant health in mind, it is central to gain a better understanding of HMO effects on the microbiome dynamics in their natural ecosystem through a holistic and ecology-inspired approach.
HMO Composition Is Linked to Infection Risk in Infants

HMOs were studied in relation with infectious diarrhea incidence in a cohort of Mexican mothers and infants \((n = 93)\) [27, 28]. Higher breast milk concentrations of \(\alpha_{1,2}\)-fucosylated HMOs were associated with a lower incidence of all-cause moderate-to-severe diarrhea. The most frequently identified cause of diarrhea in the cohort was *Campylobacter jejuni* followed by calicivirus and enteropathogenic *Escherichia coli*. Specifically, higher concentrations of 2′FL and LNFP-I in breast milk correlated with a lower incidence of *C. jejuni* and calicivirus diarrhea, respectively. These observations during the breastfeeding period did not persist in the period after breastfeeding, indicating a possible transient HMO effect in the protection from infectious diarrhea. This fits their presumed role as anti-adhesive antimicrobials. Experimental data from preclinical models also show protective effects of 2′FL from *C. jejuni* [29] and aggregating invasive *E. coli* [30]. From these data, 2′FL and other FUT2-HMOs seem to act as soluble ligands blocking *C. jejuni* from adhering to gut epithelial cells, while the protection from *E. coli* might rather be due to an anti-inflammatory effect, possibly combined with the modulation of the gut microbiota composition.

Glycans containing \(\alpha_{1,2}\)-linked Fuc expressed on epithelial cells of FUT2-positive infants could act as receptors for pathogen binding, conferring a risk to specific infectious diseases for this population [31]. Genetic studies have shown that infants and children with a nonfunctional FUT2 gene have strain-specific protection against norovirus and rotavirus [32, 33]. For specific rotavirus strains, susceptibility depends on FUT2 but also on FUT3 status [34]. Experimentally, infectivity of some rotavirus strains was reduced by the FUT2 HMO 2′FL, while other viral strains were affected by sialylated HMO, namely 3′SL and 6′SL [35]. Similarly, 2′FL also bound to specific norovirus strains [36].

Besides interfering with pathogen attachment to the host mucosa, HMOs were recently reported to exert bacterial-growth-inhibitory activities on pathogenic GBS [18, 19, 37], a major cause of sepsis in preterm infants. Growth of GBS was specifically inhibited by LNT and LNFP-I, while sialylated HMOs or galactooligosaccharides (GOS) had no effect [19]. Experimental data suggest a putative glycosyltransferase of GBS to be involved [19]. Possibly pointing to a similar mechanism, HMOs from milk of a FUT2-negative mother were shown to have bacteriostatic properties via an alteration in biofilm formation [18]. In an observation study of 183 Gambian infant-mother pairs, FUT3-positive mothers were reported to be less likely carriers of GBS, as were their infants at birth [37]. Interestingly, infants of FUT3-positive mothers were also more likely to clear GBS colonization from birth to 2–3 months of age compared to infants of FUT3-negative mothers.

In a pilot study of 49 mother-infant pairs, higher breast milk concentrations of the FUT3-HMO LNFP-II at 2 weeks were associated with a lower risk of re-
spiratory and gastrointestinal illnesses at 6 and 12 weeks in infants [38]. This association was no longer significant after the breastfeeding period. Similarly, in a nested case cohort study of 143 HIV-exposed uninfected children from Zambia, higher concentrations of fucosylated HMOs in breast milk 1 month postpartum related to a lower risk of mortality up to 2 years of age [39]. In another small mother-infant cohort from the Gambia (\(n = 33\)), higher relative breast milk concentrations of fucosylated HMO (sum of LNFP-I and LNFP-III) and concomitant lower relative abundance of LNT was associated with a lower risk of sickness up to 4 months of age [8].

For respiratory pathogens, direct HMO exposure would appear less evident, and thus any putative HMO-related protection may be mediated by the intestinal microbiome [11, 40]. Yet, experimentally, direct exposure of *Streptococcus pneumoniae* to LNnT and sialyl-LNnT and subsequent infection effectively blocked its colonization in the lung of a rabbit model [41]. In a cell-based assay, LNnT and 2′FL dose-dependently reduced influenza and respiratory syncytial virus concentrations within respiratory tract cells [42].

Observational studies together with findings from preclinical models have provided first evidence for an association between HMOs and the risk of infections, mostly in a structure-function-specific way. Mechanistically, HMOs may act through multiple functions, although preclinical models highlight specific individual functions. The current studies also provide directions to be considered in future observational studies, such as timing of milk sampling and breast milk intake, etiology of infections, quantitative versus categorical HMO analysis and finally mother and infant genetics.

**HMO Composition Might Be Linked to Allergy in Infants**

Numerous environmental, including nutrition, and genetic factors affect allergies. Among them are breast milk bioactives and possibly HMOs. In a cohort of 266 Finnish mother-infant pairs with a hereditary allergy risk, 2′FL concentrations in early breast milk associated with a lower risk to manifest IgE-associated eczema at 2 years of age only in C-section born infants [43]. This observation suggests that 2′FL may influence IgE-associated eczema through the modulation of the early-life gut microbiota, known to be different in C-section-born infants compared to vaginal-born infants. A possible relation of HMOs with cow milk allergy (CMA) was studied in another cohort of 39 mothers with infants who developed CMA by 18 months of age and 41 mothers with infants without CMA [44]. An association was seen between the milk concentration of several individual HMOs [LNFP-III, 6′SL, LNFP-I, and DSLNT (DiSialyllacto-N-tetraos)] and HMO clusters with reduced risk of CMA, with LNFP-III providing the strongest association. Breast milk sampling varied over the first 6 months after

birth and this was taken into account in the statistical analysis, because HMO concentrations change dramatically during this period. Mechanistically, the authors speculate that LNFP-III might act on the immune system via dendritic cells and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin). In a preclinical food allergy model, 2′FL and 6′SL were tested and both reduced symptoms involving mast cell activity [45].

The observational studies to date have their limitations, but still provide valuable preliminary data on possible relationships between specific HMOs and risk of allergies. To appreciate such a proposed link requires replication in larger cohorts with harmonized milk sampling, stratification for mode of delivery, and evaluation of infant FUT2 and FUT3 genotypes.

**Insight from Clinical Intervention Trials with Specific HMOs**

Recent progress in industrial biotechnology has made available few individual HMOs, namely 2′FL and LNnT. Preclinical safety toxicity tests established their safety, and both obtained approval as novel foods in the European Union and were generally recognized as safe in the USA.

In adults, both 2′FL and LNnT were studied alone or in combination at different doses from 5 to 20 g/day in a placebo-controlled, blinded, randomized trial (n = 100). Both HMOs were well tolerated and increased bifidobacterial abundance [46].

In infants, 2 placebo-controlled, blinded, randomized, clinical intervention trials showed the growth safety and tolerance of 2′FL combined with either GOS or fructooligosaccharides [47, 48; Kajizer et al., unpubl.]. Infants fed with an infant formula supplemented with 2′FL (0.2 or 1 g/L) combined with GOS or GOS alone showed similar growth as breastfed infants up to 4 months of age (n = 314). In a subgroup of infants, immune markers were measured in plasma at baseline and upon stimulation of blood cells with respiratory syncytial virus. Globally the immune profile resembled that of breastfed infants when the infant formula was supplemented with 2′FL at the lower or higher dose [48]. Another randomized controlled infant trial showed that an infant starter formula supplemented with 2 HMO, 2′FL, and LNnT (n = 88) allowed for age-appropriate growth of term born infants and was well tolerated when compared to the same infant formula without HMO (n = 87) [49]. Interestingly, secondary exploratory findings showed an association between feeding the 2-HMO infant formula and less-reported lower respiratory tract illnesses and medication use (especially antibiotics and antipyretics) during the first year of life and beyond the 6-month feeding period. At 3 months, the global microbiota profile shifted in the 2-HMO-formula-fed infants away from the control-formula-fed infants and towards that observed in breastfed reference infants. This shift was mainly due to increases in
**Bifidobacterium** concomitant with decreases in *Escherichia* and Peptostreptococcaceae [50]. A significantly higher number of infants who were fed the 2-HMO-supplemented formula showed a microbiota community structure typical for breastfed infants compared to control-formula-fed infants, who had primarily a different microbiota community structure. Interestingly, infants with a microbiota community structure typical for control-formula-fed infants had a 2 times higher risk to use antibiotics during the first year of life than those with a microbiota community typical for breastfed infants [51].

These first clinical intervention trials with specific HMOs demonstrate their growth safety and digestive tolerance. Additionally, as suggested from basic research and observational data, 2′FL and LNnT might contribute to the protection from infection-related illnesses and reduce the need for antibiotics, possibly through the modulation of the establishing early-life gut microbiota.

**Conclusion**

HMO composition is affected most notably by the maternal FUT2 and FUT3 status. This is likely due to an evolutionary selective pressure imposed by pathogens or the microbiome at large. Stage of lactation alters HMO composition possibly indicating different infant needs at different extrauterine developmental stages. However, giving birth to a preterm or term infant, who are at different developmental stages, seems not to affect the HMO composition of breast milk. Clinical observations corroborated by preclinical data and clinical intervention trials support a role for specific HMOs in immune protection, primarily from infection-related morbidity and use of antibiotics. Further clinical studies, well-designed observational studies, and especially placebo-controlled interventions are warranted to further substantiate and grow our understanding of the HMO biology and significance for infant nutrition.

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