

## **HUMAN MILK OLIGOSACCHARIDES (HMO) AS PRIMERS OF THE INFANT MICROBIOME**

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### **SUMMARY**

Human milk oligosaccharides (HMO) are a group of more than 150 different complex sugars that are highly abundant in human milk, but currently not present in infant formula. HMO composition follows a basic blueprint, but every woman generates a distinct HMO profile. These inter-individual differences are at least in part genetically determined. Whether or not other maternal genetic or environmental factors contribute to HMO biosynthesis remains unknown. HMO prime the infant microbiome through multiple direct and indirect mechanisms. HMO are human milk prebiotics that serve as metabolic substrates for potentially beneficial bacteria in the infant gastrointestinal tract and, thus, help shape the infant gut microbiome. HMO act as soluble decoy receptors that block the attachment of viral, bacterial or protozoan parasite pathogens to epithelial cell surface glycans and have the potential to reduce pathogen colonization and invasion and prevent infectious diseases in the gut and, potentially, also the respiratory and urinary tracts. HMO are antimicrobials that act as bacteriostatic or bactericidal agents. In addition to these direct microbiome-priming effects, HMO also affect host epithelial cell and immune cell responses that indirectly prime the microbiome. While most research currently focuses on potential health benefits for the breast-fed infant, little is known about potential benefits for the breast-feeding mother. In addition, HMO are found in the urine of pregnant women as early as at the end of the first trimester, suggesting that HMO might also affect pregnant women and the growing foetus. Carefully selected preclinical *in vitro* and animal models in combination with mother-infant cohort studies will enable us to investigate the potential benefits of individual structurally defined HMO.

### **INTRODUCTION**

Human Milk Oligosaccharides (HMO) are unconjugated complex sugars (carbohydrates, glycans) that are highly abundant in human milk, but not in infant formula (reviewed in Bode, 2012). One litre of mature human milk contains 10-15 g HMO, which often exceeds the total amount of protein and is 100- to 1,000-fold higher than the concentration of oligosaccharides in bovine milk, the basis of most infant formula. HMO concentrations are even higher in human colostrum, the fluid secreted by the mammary gland during the first few days of lactation. HMO consist of the five

monosaccharide building blocks galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc) and the sialic acid (Sia) derivative N-acetylneuramic acid. HMO composition follows a basic blueprint. All HMO carry lactose (Gal $\beta$ 1-4Glc) at the reducing end, which can be elongated in  $\beta$ 1-3-linkage by two different disaccharides, either Gal $\beta$ 1-3GlcNAc (type 1 chain) or Gal $\beta$ 1-4GlcNAc (type 2 chain). HMO can be branched if a Gal/GlcNAc disaccharide attaches in  $\beta$ 1-6-linkage. HMO with more than 15 disaccharide units have been described, forming complex structural backbones. Structural diversity increase further by the addition of Fuc and Sia.

Fuc can be added to the HMO backbone in  $\alpha$ 1-2-,  $\alpha$ 1-3- or  $\alpha$ 1-4-linkage. HMO fucosylation is highly dependent on the mother's Lewis blood group status (Kumazaki and Yoshida, 1984; Johnson and Watkins, 1992; Chaturvedi et al., 2001; Stahl et al., 2001). Fucosyltransferase 2 (FUT2) catalyses the addition of Fuc in  $\alpha$ 1-2-linkage on Lewis blood group epitopes as well as on HMO (Kumazaki and Yoshida, 1984). FUT2 is actively expressed in over 70% of the population (Secretors). Milk of Secretor women contains high concentrations of  $\alpha$ 1-2-fucosylated HMO. 2'-fucosyllactose (2'FL) is one of the dominant HMO in the milk of secretor women. Non-secretors, however, do not express an active FUT2 and the milk of Non-secretor women lacks  $\alpha$ 1-2-fucosylated HMO. 2'FL is almost completely absent. Fucosyltransferase 3 (FUT3) on the other hand catalyses the addition of Fuc in  $\alpha$ 1-3/4-linkage (depending on the type of the underlying HMO backbone), and FUT3 can also be inactive in parts of the population (Lewis negative) (Johnson and Watkins, 1992). The milk of Lewis negative women has markedly reduced  $\alpha$ 1-3/4-fucosylated HMO, although

they are not completely absent because another fucosyltransferase covers some redundant activity. Depending on the expression of active FUT2 and FUT3 enzymes, women can be separated into four groups: 1. Lewis positive Secretors (FUT2 active, FUT3 active), 2. Lewis negative Secretors (FUT2 active, FUT3 inactive), 3. Lewis positive Non-secretors (FUT2 inactive, FUT3 active), and 4. Lewis negative Non-secretors (FUT2 inactive, FUT3 inactive). Accordingly, the oligosaccharide composition in the milk of women from these four groups varies significantly (Chaturvedi et al., 2001; Stahl et al., 2001).

Sia can be added to the HMO backbone in  $\alpha$ 2-3- or  $\alpha$ 2-6-linkage. Sia contains a carboxyl-group, which, at physiological pH, introduces a negative charge to HMO. Therefore, sialylated or acidic HMO carry one or more negative charges depending on the number of Sia linked to the HMO backbone.

HMO synthesis is in part genetically determined, which is highly apparent with the differential fucosylation between Secretors and Non-secretors described earlier. Genes other than FUT2 and FUT3 that contribute to chain elongation, branching or sialylation might be differentially expressed in different women and lead to a distinct HMO composition. Whether or not environmental factors like nutrition or use of medication and drugs impacts HMO synthesis remains to be elucidated.

In summary, human milk, unlike the milk of most other mammals, contains very high concentrations of a structurally diverse group of more than 150 different complex sugars called human milk oligosaccharides (HMO). HMO composition follows a basic blueprint, but each woman produces a distinct profile of different HMO at different concentrations that can change over the course of lactation. These inter- and

intra-individual differences in HMO composition are in part determined by genetics.

Once ingested by the breast-fed infant, HMO resist the low stomach pH as well as degradation in by pancreatic and brush border enzymes (*Gnoth et al.*, 2000; *Engfer et al.*, 2000). Approximately, 1% of the ingested HMO are absorbed in the intestine reach the infant's blood, and are excreted intact in the infant's urine (*Rudloff et al.*, 1996, 2006, 2011). The majority of HMO is either metabolized by the infant's gut microbes or excreted intact with the

infant's faeces (*Albrecht et al.*, 2010, 2011). Consequently, HMO are only found in the urine and faeces of breast-fed, but not formula-fed infants. While this article focuses on HMO as primers of the infant microbiome, it is important to emphasize that HMO are not contained in the infant's gut and potentially reach all organs, including the liver and the brain, as well as the respiratory and the urinary tract. Therefore, HMO effects may not be localized to only the gut, but impact the infant on multiple different levels, which may or may not involve microbes.

## PREBIOTICS

A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota, that confers benefits upon host well-being and health (*Roberfroid et al.*, 2007). HMO serve as metabolic substrates for specific bacteria (e.g. *Bifidobacterium longum* subsp. *infantis*; *B. infantis*, JCM1222). As a consequence, these bacteria have a growth advantage and thrive. Other bacteria that cannot utilize HMO have a disadvantage and do not grow as well or not at all. Thus, HMO are the first prebiotics that humans encounter with their diet. The ability to utilize HMO requires an entire set of enzymes, transporters and other molecules. Certain bacteria have evolved together with HMO and express sialidases to cleave Sia or fucosidases to cleave Fuc to name just a few examples. Only very few bacteria are capable of degrading the entire array of HMO (*LoCasio et al.*, 2007; *Marcobal et al.*, 2010; *Asakuma et al.*, 2011). Other bacteria may only be able to utilize parts of HMO. For example, bacteria with a certain fucosidase may be able to utilize the Fuc, but not Sia or the underlying backbone. Some

bacteria may be able to utilize HMO only after other bacteria have removed the Fuc from the backbone, creating a "community feast" where multiple different bacteria may be able to degrade the entire array of HMO, but only when they act together as a community.

While the sequential degradation of HMO by different microbes needs to be further elucidated, it is evident that, based on their structural diversity, different HMO can be metabolized by different bacteria. In other words, not all HMO lead to the same changes in composition and/or activity in the gastrointestinal microbiota and have the same benefits upon host well-being and health. Prebiotic effects are likely structure-specific, raising the question whether or not we can use structurally distinct HMO to elicit a certain response in microbiota composition. Targeted microbiome changes with specific HMO or other glycans might not only be desirable for the neonate, but also for adolescents and adults that suffer from diseases like obesity or inflammatory bowel disease that have been shown to be associated with dysbiosis.

## ANTI-ADHESIVES

HMO are more than “food for bugs”. Many viral, bacterial or protozoan parasite pathogens need to attach to epithelial surfaces in order to proliferate and in some cases invade and cause disease. In many cases the initial attachment is to epithelial cell surface sugars (glycans) also known as the glycocalyx. While these glycans are often conjugated to proteins or lipids, HMO resemble some of the same glycan structures and serve as soluble decoy receptors that block pathogen binding to epithelial cells. Unbound pathogens can no longer attach and are washed out without causing disease. Norovirus and Rotavirus are examples for viral pathogens that bind to the epithelial glycocalyx; HMO resemble the glycan binding partners and block viral attachment, providing one explanation for the reduced incidence of these viral infections in breast-fed infants compared to formula-fed infants. *Campylobacter jejuni* (Ruiz-Palacios et al., 2003) and enteropathogenic *E. coli* (Manthey et al., 2014) are examples for bacterial pathogens that follow the same principal and have significant impact on infant health as they are responsible for a majority of bacterial diarrheal episodes. Our lab has recently shown that HMO also prevent the attachment of the protozoan parasite *Entamoeba histolytica* (Jantscher-Krenn et al., 2012). While rather uncommon in the US or Europe,

*E. histolytica* infects more than 50 million people worldwide and causes the disease amoebiasis, leading to more than 100,000 death annually (Pritt and Clark, 2008). *E. histolytica* expresses a lectin, a glycan-binding protein, which is a major virulence factor involved in *E. histolytica* attachment to as well as killing and phagocytosis of intestinal epithelial cells (Cano-Mancera and Lopez-Revilla, 1987; Saffer and Petri, 1991). HMO block the lectin and prevent attachment, killing and phagocytosis. The effects are structure-specific and require a terminal Gal on the HMO to be effective. Fucosylation of the terminal Gal abolishes the effect.

A growing body of evidence from tissue culture attachment assays and animal infection models support the notion that HMO are structure-specific anti-adhesives. However, well-designed and fully-powered mother-infant cohort studies and, most importantly, intervention studies are required to confirm that individual HMO or a mixture of different HMO reduce the incidence of infectious diseases caused by viral, bacterial and protozoan parasite pathogens. If confirmed, HMO could become urgently needed alternatives or additions to currently existing antibiotics that alone become more and more ineffective as pathogens develop antibiotic resistance.

## ANTIMICROBIALS

HMO may protect us from pathogens not only by serving as anti-adhesives at the interface of microbe-host interactions. HMO may have a more direct way of making a pathogen’s life difficult. In collaboration with Victor Nizet’s lab at the University of California, San Diego, we have recently shown

that *Streptococcus agalacticae* (Group B Streptococcus; GBS) is no longer able to proliferate when HMO are present (Lin et al., manuscript in preparation). GBS is one of the leading neonatal pathogens affecting about 1 in 2,000 new-borns in the U.S. An estimated 15-40% of all pregnant women

are colonized with GBS in the gastrointestinal or genital tract (Anthony et al., 1981; Campbell et al., 2000); more than half of them experience miscarriage or stillbirth and 16-53% of colonized mothers may pass the bacteria onto their babies during childbirth (Phares et al., 2008; Muller et al., 2006). Infected infants can develop sepsis, pneumonia, and life-threatening meningitis. GBS colonization in the genital tract also increases the probability of urinary tract infections (UTIs) in pregnant women (Muller et al., 2006). We used multidimensional chromatography and identified specific non-sialylated HMO as having the most pronounced bacteriostatic effect on GBS. We also used a GBS transposon library and identified a GBS mutant that is no longer susceptible to the bacteriostatic effects of HMO. The mutant lacks a gene that encodes a glycosyltransferase and additional *in vitro* studies suggest that GBS employs the glycosyltransferase to incorporate specific HMO into their cell membrane, which then stops GBS proliferation similar to some of the commercially available antibiotics. In fact, HMO synergize with antibiotics like vancomycin or ciproflaxacin and significantly improve their IC<sub>50</sub> *in vitro* (Lin et al., manuscript in preparation). These most recent results are very promising in developing new antibiotics that are based on natural compounds like HMO and synergize with already available

antibiotics that, when used alone, start to lose efficacy due to the development of antibiotic resistance.

HMO may not only protect from viral, bacterial or protozoan pathogens. Our most recent work in collaboration with Dr. Cheryl Gale's group at the University of Minnesota suggests that HMO also affect fungal-host interactions (Gonia et al., 2015). *Candida albicans*, a prevalent fungal colonizer of the neonatal gut (Saiman, 2001; La Tuga, 2011; Heisel, 2015), causes the overwhelming majority of invasive fungal disease in premature infants and is highly associated with concurrent diagnoses of necrotizing enterocolitis and focal intestinal perforation, life-threatening intestinal disorders (Coates, 2005; Ragouilliaux, 2007). Treatment with HMO significantly reduced invasion of human premature intestinal epithelial cells (pIECs) by *C. albicans* in a dose dependent manner (Gonia et al., 2015). The decreased invasive potential of *C. albicans* correlated with a delay in hyphal growth and morphogenesis as well as a reduction in the ability of *C. albicans* to associate with pIECs, processes important for the initial pathogenesis steps of *C. albicans* infections. Again, HMO appear to directly affect the microbe, here the fungus *C. albicans*, altering hyphal growth and morphogenesis, which then makes it more difficult for the pathogen to attach, invade and cause disease.

## ALTERING HOST CELL RESPONSES

HMO may not only impact microbes directly, but also indirectly by altering host cell responses. HMO have been shown to modulate intestinal epithelial cell apoptosis, proliferation and differentiation (Kuntz et al., 2008). HMO have also been shown to alter intestinal epithelial cell gene expression leading

to changes in the cell surface glycocalyx (Angeloni et al., 2005). Thus, HMO may not only affect microbe-host attachment by serving as soluble decoy receptors as described above, but also by changing the expression of the glycocalyx receptors by reprogramming the epithelial cell. Our own lab recently

set out to investigate whether or not HMO can serve as anti-adhesives not only for bacteria in the infant gut, but also for bacteria in the urinary tract (Lin et al., 2014). To our surprise we found that HMO indeed reduce invasion of uropathogenic *E.coli* (UPEC), but not by serving as anti-adhesives that reduce UPEC attachment to epithelial cells. Instead, HMO interact with the epithelial cells and make them more resistant against UPEC attacks. HMO strongly suppress intracellular signaling of apoptotic pathways that renders the epithelial cell irresponsive when UPEC tries to destroy them. The effects are highly structure-dependent and only sialylated HMO like 3'-sialyllactose are effective (Lin et al., 2014).

HMO not only alter epithelial cell responses. HMO also affect immune cell responses. For example, we have recently shown that specific sialylated

HMO reduce the expression of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in LPS-stimulated macrophages, both of murine and human origin (Autran et al., manuscript in preparation). This and other examples from other immune cell types (Eiwegger et al., 2004, 2010) suggest that HMO alter immune responses to pathogens, adding to the repertoire of mechanisms by which HMO interfere with microbe-host interactions and contribute to the protection of the breast-fed infant.

In summary, HMO interfere with microbe-host interactions not only directly by serving as prebiotics, anti-adhesives or antimicrobials, but also indirectly by altering epithelial and immune cell responses that set the stage for microbe-host interactions and help shape microbiota and microbiome compositions.

## HMO AS PRIMERS OF THE MOTHER'S MICROBIOME

HMO may not only prime infant microbiomes. Milk, for example, is not sterile and harbours microbial communities (Hunt et al., 2011). When milk is synthesized and temporarily stored in the alveoli and ducts of the mammary gland, HMO are in contact with the milk microbiota and may shape the microbiome by similar mechanism as described for the infant gut microbiome. HMO may serve as metabolic substrates for specific milk microbes and help them thrive. HMO may act as antimicrobials with bacteriostatic or bacteriocidal effects that contain the growth of microbes. HMO may have anti-adhesive effects and prevent bacterial attachment to mammary gland cells surfaces, which may impact bacterial growth. HMO may also have direct effects on epithelial cells or immune cells in the mammary gland, which helps set the environment for certain

bacteria to thrive while others are kept in check.

In collaboration with Shelley and Mark McGuire at Washington State University and the University of Idaho we have recently shown strong associations between HMO composition and milk microbiota composition and identified an additional mechanism through which HMO impact microbes (Hunt et al., 2012). We found that high concentrations of the HMO 2'FL correlate with high concentrations of *Staphylococcus aureus* in mother's milk. In fact, *S. aureus* grows better in media containing the HMO 2'FL, suggesting the bacteria utilize HMO as a metabolic substrate. To our surprise, HMO concentrations did not decrease when *S. aureus* propagated. Instead, we found that the concentrations of several amino acids decline when *S. aureus* grows on HMO, suggesting that HMO

trigger a metabolic switch that allows the bacteria to utilize specific amino acids more efficiently (Hunt et al., 2012).

HMO may not only prime the mother's milk microbiome. Already in the late 1970s, studies revealed that HMO appear in the urine of pregnant women as early as at the end of the first trimester, long before milk is secreted for infant consumption (Hallgren et al., 1977; Hallgren and Lundblad, 1977). These observations are now being confirmed and the data is rapidly expanding. If HMO appear in the urine of pregnant women, do they help shape the urinary tract or vaginal microbiome? Urinary tract infections in pregnant women can lead to ascending infections and cause preterm delivery. Are some women at a higher risk because they lack specific HMO during pregnancy? First reports show that HMO are present in the blood of

pregnant women. Do they cross the placental barrier? Do they appear in cord blood and in amniotic fluid? Do HMO impact foetal growth and development long before they are made available to the neonate through mother's milk? Do they shape the women's microbiome in niches other than urinary and reproductive tract? Do they have systemic effects in pregnant women? Studies are on the way to describe HMO composition, concentration and inter- and intra-individual variations in different fluids and tissues in pregnant women. Results from these studies will lay the foundation for mother-infant-cohort studies that, in combination with suitable preclinical models, will guide us in elucidate the role of HMO as primers of the mother's microbiome and help us understand how HMO benefit the pregnant woman and the growing foetus long before milk is secreted.

## THE POWER OF COHORT STUDIES

Most of the data reviewed so far stem from *in vitro* studies in the test tube or in tissue culture or from *in vivo* studies in animal models. The gap between these preclinical studies and clinical intervention studies in humans is immense, carrying the risk that results generated in tissue culture or animal models don't translate to benefit the human neonate. Mother-infant cohort studies are a powerful way to narrow this gap between preclinical and clinical studies. Let's assume preclinical models suggest that HMO reduce a certain disease. Let's further assume that a multidimensional chromatography approach identifies a structurally distinct oligosaccharide within the HMO that is responsible for the beneficial effect. Now, let's assume that mother-infant cohort studies reveal that high concentrations of the oligosaccharide in

mother's milk are associated with a lower incidence of the disease in infants. The combination of these results would be very informative in designing an intervention study to confirm that the identified oligosaccharide indeed reduces the disease in question. Pre-clinical models could continue to help elucidate the underlying mechanisms, and newly developed synthetic approaches could make the specific HMO available to enable clinical studies and application. Due to the immense inter- and intra-individual variation in HMO composition, these cohort studies often require the recruitment of hundreds of mother-infant pairs and the collection and analysis of hundreds and thousands of milk samples. However, until recently HMO analysis was tedious and expensive. For example, in 2012 our lab published results from a study that

investigated associations between HMO composition and mother-to-child HIV transmission via breastfeeding (Bode et al., 2012). At that time, we were able to analyse 10-12 samples per week for a total of about 100 milk samples. Conducting cohort studies that analysed the HMO composition of hundreds or thousands of milk samples was simply unfeasible. In the last couple of years, our lab as well as a couple of other labs have developed new rapid high-throughput methods that allow HMO composition analysis from as little as 10  $\mu$ L milk usually in 96-sample format in parallel in within days (Autran et al., manuscript in preparation). In addition, our HPLC-based method uses an internal standard from the very beginning of sample preparation that allows for absolute HMO quantification, which has often been a

limitation, especially in most mass spectrometry-based methods. These new analytical methods now enable us to conduct large mother-infant cohort studies with the aim to investigate how specific HMO are associated with health and disease of the infant, but also the mother. For example, HMO composition can now be linked to infant gut microbiota and microbiome composition, risk for infants to develop necrotizing enterocolitis (Autran et al., manuscript in preparation) or acquire viral or bacterial infections, or risk for mothers to develop urinary tract infections and deliver their baby prematurely. In addition, the methods now also allow us to study how maternal factors like genetics, health status, nutrition, medication or drugs impact HMO composition.

## CONCLUSIONS

Future studies are going to show how individual, structurally defined HMO or mixtures of different HMO benefit the breast-fed infant and the breastfeeding mother, but also pregnant women and the growing foetus. It is important to emphasize that HMO are a group of at least 150 different and structurally distinct oligosaccharides. It is going to be essential to clearly define structure-function relationship in the context of different health states and diseases. Preclinical models have to be carefully selected to generate

meaningful data that translates to humans. Multidimensional chromatography is going to be a helpful tool to identify individual and structurally well characterized HMO that are responsible for observed beneficial effects. Mother-infant cohorts are going to provide valuable data to help close the gap between preclinical models and clinical intervention studies. Future research is going to reveal what factors drive HMO composition and how HMO composition impacts health outcomes, in part via priming microbiomes.

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