Protective Factors in Human Milk

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Human Milk Oligosaccharides: Structure and Functions

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Abstract

Oligosaccharides are a group of complex glycans that are present in the milk of most mammals. However, human milk is unique as the concentrations of human milk oligosaccharides (HMOs) are much higher than those of other mammals, and their structural composition is more complex and varies between women. These observations prompt several questions: (i) Why are humans unique when it comes to milk oligosaccharides? (ii) Which maternal genetic and environmental factors drive the interindividual variation in HMO composition? (iii) What are the short- and long-term health benefits for the infant – and potentially also the mother? The combination of genome-wide association studies, milk transcriptomics, in vitro gene editing, and in silico pathway modeling allows us to reconstruct HMO biosynthetic pathways. Using new data mining approaches and leveraging samples and metadata from large mother-infant cohorts enable us to identify associations between HMO composition and infant and maternal health outcomes. Suitable preclinical models and clinical intervention studies allow us to corroborate the established associations for causal relationships and test for in vivo efficacy in humans. Knowledge generated from these different approaches will help us establish true structure-function relationships and provide the rigorous evidence required to improve infant health and development.

What Are Human Milk Oligosaccharides?

Oligosaccharides (from the Greek ὀλίγος olígos, "a few," and σάκχαρ sácchar, "sugar") are saccharide (sugar) polymers containing a small number (typically 3–10 or more) of monosaccharides (simple sugars). Unlike the milk of most other mammals, human milk is unique as it contains a variety of more than 150 different and structurally distinct oligosaccharides at high concentrations. In fact, with 5–15 g/L, the total concentration of human milk oligosaccharides (HMOs) in mature milk often exceeds the total concentration of human milk proteins, making HMOs the third most abundant component after the simple milk sugar lactose and lipids, and not counting water [1].

HMOs contain up to 5 different building blocks (monosaccharides): glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (Sia). Different HMOs are generated depending on which and how many of these building blocks are used, and how they are linked together [1]. Figure 1a shows the blueprint of the HMO structure assembly. All HMOs carry lactose (Galβ1–4Glc) at the reducing end. Lactose can be elongated by the addition of the disaccharides lacto-N-biose (Galβ1–3GlcNAc) or N-acetyllactosamine (Galβ1–4GlcNAc). Lactose or the elongated chains can be modified with sialic acid in α2–3- or α2–6-linkage and/or fucosylated in α1–2-, α1–3-, or α1–4-linkage, vastly expanding the diversity of the HMO structure portfolio. For example, each sialic acid monosaccharide contains a carboxyl group and introduces a negative charge to the HMO molecule altering its structural properties. The HMO structure often determines its functions [2].

Although the HMO composition follows a basic blueprint and more than 150 different HMOs have been identified so far, it is important to note that every woman synthesizes and secretes a distinct HMO composition profile that varies substantially between different women (Fig. 1b) but remains fairly constant over the course of lactation for the same woman [3]. So far, our lab has analyzed the HMO composition in more than 10,000 milk samples collected from women around the world as part of various collaborative projects. Figure 1c presents some of the data in a principal component (PC) plot, highlighting once again that HMO composition profiles vary between women, but also that there are distinct HMO profile clusters or HMO *lactotypes*.

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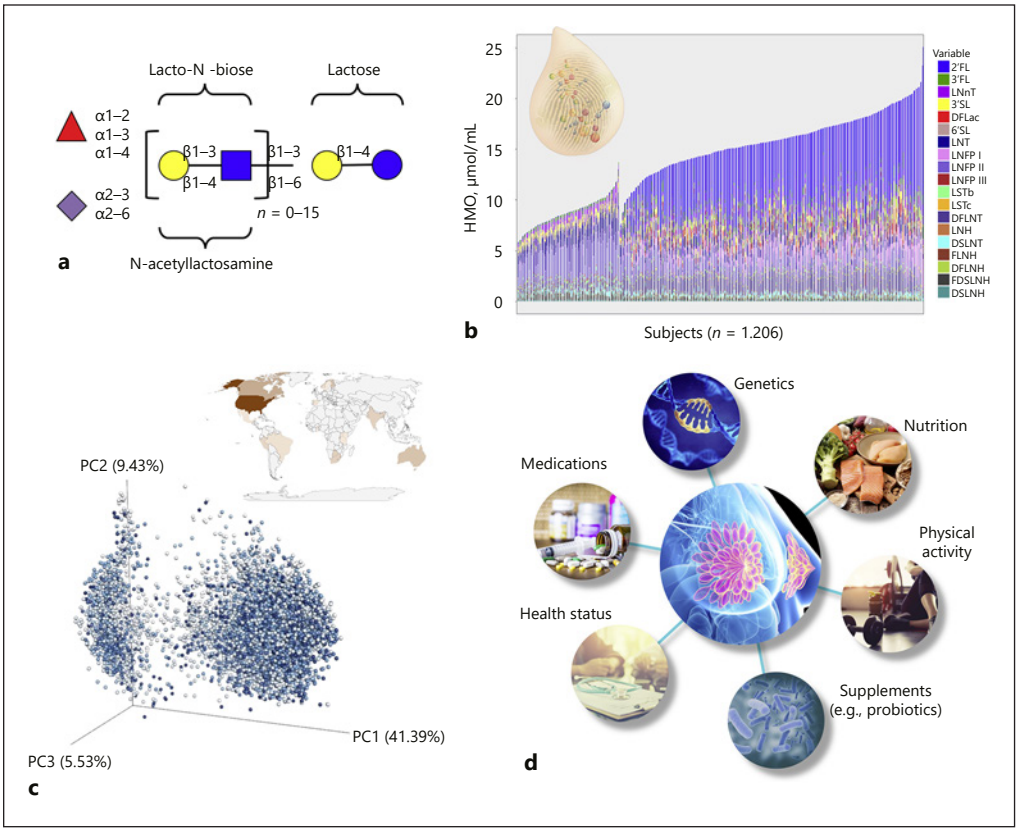

Fig. 1. Human milk oligosaccharide (HMO) composition varies between mothers, which is driven by genetics as well as environmental modifiers. **a** HMOs consist of the 5 monosaccharide building blocks: glucose (Glc, blue circle), galactose (Gal, yellow circle), N-acetylglucosamine (GlcNAc, blue square), fucose (Fuc, red triangle), and N-acetylneuraminic acid (NeuAc, purple diamond), and the HMO structural composition follows a basic blueprint. **b** HMO composition varies between mothers as exemplified by data from 1,206 mothers in the CHILD cohort [3]. Each bar on the *x*-axis represents a milk sample, each color is a specific HMO with concentrations indicated on the *y*-axis. **c** Principal component (PC) analysis of the HMO composition in over 10,000 milk samples collected from around the world shows how different HMO *lactotypes* cluster in different areas of the 3-dimensional space. Each dot in the space represents the HMO composition of a separate milk sample. The closer the dots are in the space, the more similar the HMO composition between the samples. The farther apart the dots, the more different the HMO composition in the samples. The left/right clustering is mostly driven by single nucleotide polymorphisms in the gene encoding for the enzyme fucosyltransferase 2 (FUT2) which catalyzes the addition of fucose in α1–2-linkage. **d** In addition to genetics, many other maternal factors drive the observed variation in HMO composition, many of them not well studied and the underlying mechanisms poorly understood.

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What Drives the Variation in Human Milk Oligosaccharide Composition?

Figure 1d showcases just a few of the potential drivers of HMO composition. Genetics appears to be one of the strongest determinants of HMO composition. In fact, most of the drastic left/right clustering along the PC1 axis in the PC plot in Figure 1c can be explained by a single nucleotide polymorphism (SNP). In other words, the difference in 1 bp out of the approximately 3 billion bp of the human genome dramatically alters the overall oligosaccharide composition of human milk, establishing the so-called secretor and nonsecretor *lactotypes*. The affected gene encodes the enzyme fucosyltransferase 2 (FUT2), which catalyzes the addition of fucose to lactose or the elongated HMO chain in an α1–2-linkage [4]. Specific SNPs introduce a premature stop codon in the FUT2 reading frame, leading to incomplete FUT2 enzyme synthesis and lack of function. While the milk of women with active FUT2 (secretors) contains high amounts of α1–2-fucosylated HMOs like 2′-fucosyllactose (2′FL) or lacto-N-fucopentaose (LNFP) 1, these specific HMOs are almost completely absent in milk of women with inactive FUT2 (nonsecretors) [5]. Lack of FUT2 activity has ripple effects throughout the entire HMO biosynthetic pathway and impacts the concentration of almost all other HMOs, not just the ones that are α1–2-fucosylated. Similar, but slightly more subtle effects can be observed with SNPs in the gene encoding the enzyme fucosyltransferase 3 (FUT3) that is linked to the Lewis blood group antigen and catalyzes the addition of fucose to lactose or the elongated HMO chain in an α1–3- or α1–4-linkage [6]. Presence or lack of FUT3 activity establishes the Lewis-positive or -negative *lactotypes*.

FUT2 and FUT3 are only 2 of the enzymes involved in HMO biosynthesis. Most of the other biosynthetic steps and catalyzing enzymes remain poorly characterized, leaving almost an entire biosynthetic pathway in human biology to be discovered. Remarkably, the complete pathway is only activated in humans, only in females, only in the mammary gland, and only during pregnancy and lactation. This specific restriction to species, gender, tissue, and time makes the pathway difficult to study, but the challenge may hold great opportunities to better understand the uniqueness of human biology. We are currently employing a combination of genome-wide association studies, human milk transcriptomics, HMO-omics as well as in silico pathway modeling and in vitro target validation to unravel HMO biosynthesis in the human mammary gland.

In addition to genetics, environmental, modifiable factors like maternal diet and physical activity, use of supplements, maternal health status, and use of medications during pregnancy and lactation may also affect HMO composition [3]. For example, our first data from animal models show that high-fat diet lowers the amount of mouse milk oligosaccharides while physical activity raises it

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[manuscript submitted]. Other data from our team show that women who use a combination probiotic during pregnancy have significantly different HMO composition than women who served as controls and did not receive the probiotic [7]. Whether maternal health conditions like obesity, gestational diabetes, or chronic inflammatory diseases affect HMO composition remains largely unknown and is currently an area of active investigation.

What Happens to Human Milk Oligosaccharides after Ingestion?

Once ingested, HMOs resist the low stomach pH as well as degradation through pancreatic and brush border enzymes in the small intestine [8, 9], with the potential exception of type 2 chains in which the terminal β1–4-linked Gal may be cleaved off by the enzyme lactase. Approximately 1% of the ingested HMOs are absorbed and can be measured in the systemic circulation as well as in the urine [10, 11], indicating that HMO effects extend to tissues and organs other than the intestine. Most HMOs reach the distal small intestine and colon intact, where they are either metabolized by microbes or excreted with the feces.

What Are Potential Human Milk Oligosaccharide Functions?

HMOs are often considered as human milk prebiotics, serving as metabolic substrates for potentially beneficial bacteria in the infant gut and as such shaping a *healthy* microbiome [12]. However, we strongly believe that HMOs are more than just *food for bugs*. In fact, it is believed that oligosaccharides originally evolved to serve the opposite purpose: not to feed bacteria but to keep them from growing [13]. *Milk* – or its evolution ancestor – is believed to have developed as a secretion to keep eggs moist. With the moisture came the risk of bacterial and fungal growth and contamination, which required the development of antimicrobial components, and oligosaccharides were likely part of that antimicrobial defense system. Although lactose is an integral structural part of all HMOs, lactose itself likely developed later during evolution, maybe as additional energy source. Thus, from the very beginning, HMOs developed as antimicrobials, and their additional prebiotic effects likely evolved much later. Research has shown that specific HMOs serve as bacteriostatic molecules that stop the growth of bacteria like group B streptococci [14]. HMOs also serve as antiadhesives, mimicking epithelial cell surface receptors used by many viruses, bacteria, and protozoan parasites to attach to host surfaces as a requirement for microbes to find their niche, proliferate, and in some cases invade and cause disease [1]. Thus, HMOs are soluble decoy receptors that prevent

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microbes from binding to epithelial cells. As a consequence, potential pathogens are unable to attach, proliferate, and cause disease [1]. Antiadhesive effects of HMOs have been described for *Campylobacter jejuni* strains in vitro and in animal models [15], and the same HMOs are associated with lower *Campylobacter* diarrhea in a mother-infant cohort [16]. We have since been able to identify specific HMOs to also block enteropathogenic *Escherichia coli* (EPEC) adhesion and lesion formation in tissue culture as well as in mice [17; unpublished data].

Prebiotic or antimicrobial? Those effects do not have to be mutually exclusive. Commensals like certain *Bifidobacteria* seem to prefer simple, low-molecular-weight HMOs [18], whereas the antimicrobial properties seem to depend on higher-molecular structures. Smaller HMOs like fucosyllactoses, sialyllactoses, or lacto-N(neo)-tetraoses may serve as *food for bugs*, be preferentially utilized as metabolic substrates, and therefore protect the higher-molecular-weight HMOs from being degraded and available to exert their antimicrobial properties.

However, evolution continues, and certain microbes may have started to exploit human milk components to their advantage. We have recently shown that a specific rotavirus strain with a G10P [11] spike protein increases its infectivity in tissue culture assays in the presence of specific HMOs [19]. The same HMOs are at higher concentrations in the milk of women whose infants develop symptomatic rotavirus infections. Are pathogens getting ahead in the host-microbe *arms race*? Or can we leverage the gained knowledge to develop new vaccination strategies? Rotavac, a live attenuated vaccine against rotavirus, also increases its infectivity in tissue culture assays in the presence of the identified HMOs, pointing to new opportunities to include HMOs to boost vaccination success.

In addition to modifying host-microbe interactions, HMOs are associated with infant growth and body composition. We have shown that specific HMOs associate with infant weight, lean mass, and fat mass in 25 mother-infant dyads in the US [20]. Employing the same HMO analytical platform, we have identified specific HMOs that are positively or negatively associated with excessive weight gain in exclusively breastfeed infants in 30 mother-infant dyads in Denmark [21]. Remarkably, lacto-N-neo-tetraose (LNnT) was negatively associated with body fat and body weight in both studies, and 2′FL was positively associated with infant weight gain. While these are rather small cohorts, we have recently completed the analysis of HMO composition in a larger cohort study including 802 mother-infant dyads in Finland [manuscript submitted], and the very same HMOs were significantly associated with infant weight gain all the way out to 5 years of age, long after breastfeeding occurred, suggesting long-term effects on infant growth and body composition. It is important to note that the data stem from association studies and do not allow us to draw conclusions on cause-and-effect relationships, but the observations from these 3 different

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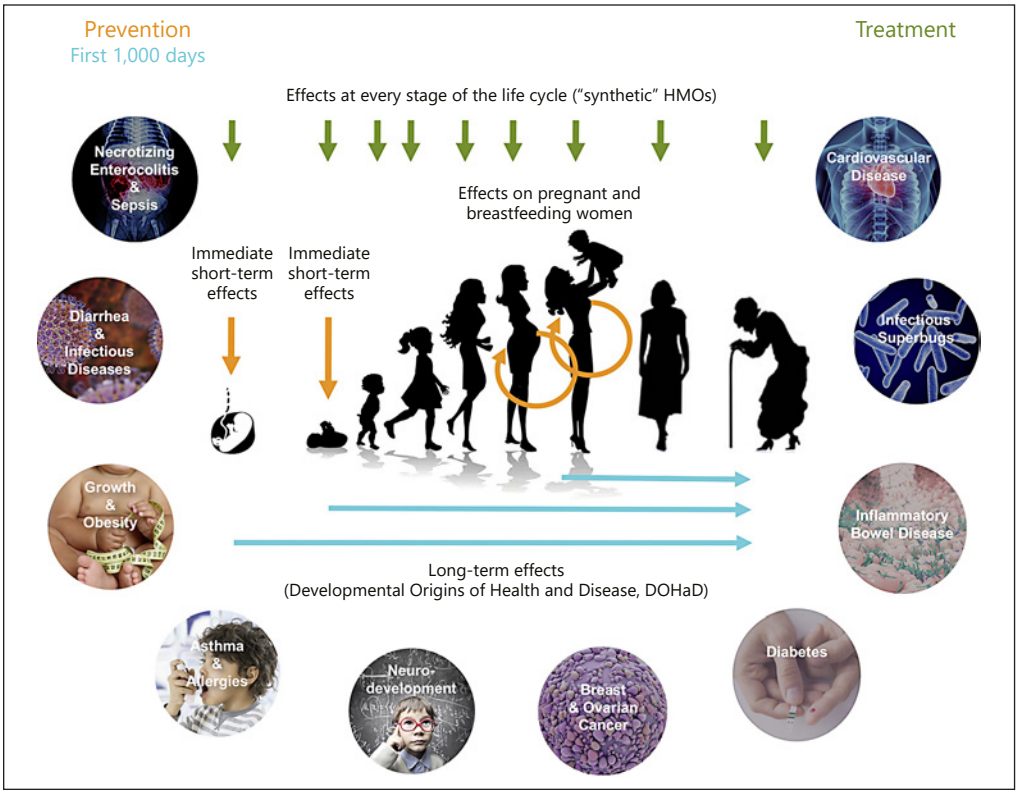

Fig. 2. The role of human milk oligosaccharides (HMOs) throughout life. HMOs have the potential to affect health and development at all stages of the life cycle. At one end of the spectrum, HMOs already appear in the amniotic fluid with potential immediate as well as long-term effects on the fetus. At the other end of the spectrum, HMOs reduce chronic inflammation and may serve as novel therapeutics to prevent or treat patients with heart attacks or stroke caused by atherosclerosis.

studies drive the need to understand causalities and underlying mechanisms, and may provide opportunities to use HMOs to support infant growth and weight gain when needed.

Can We Harness the Power of Human Milk Oligosaccharides to Develop New Therapeutics for Adults?

Rapidly accumulating data strongly suggests that HMOs have immediate benefits for infants with potential long-lasting effects throughout life, adding to the concept of Developmental Origins of Health and Disease (DOHaD) and the im-

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portance of the first 1,000 days. Recently, we have shown that HMOs already appear in the amniotic fluid [22], suggesting they may also affect fetal health and development, again with potential long-lasting effects for life. However, HMOs may not only be good for infants, they may also affect maternal immediate and long-term health. HMOs appear in the maternal circulation as early as at the end of the first trimester of pregnancy and are excreted intact in maternal urine all throughout lactation [23].

Last, but certainly not least, HMOs now become available at large scale and fairly low cost, mostly because of recent advances in bioengineering microbes that utilize simple sugars to synthesize complex HMOs [24]. These *synthetic*, but structure-identical HMOs are now added to first infant formula products, but their application outside the maternal-infant space is also explored. First studies indicate the use of specific HMOs like 2′FL as novel therapeutics to improve gut health, and we have recently identified specific HMOs that accelerate macrophage resolution and reduce chronic inflammation in cell culture, and significantly reduce arthritis and atherosclerosis in an animal model [manuscripts submitted]. Most importantly, HMOs are natural components that are highly abundant in human milk and fed to infants every 2–3 h for several months. HMOs evolved to be safe and support infant immediate and long-term health and development, and they are likely safe for application in adults as well, opening new opportunities to develop HMOs as novel therapeutics for some of today's most common, painful and often deadly diseases. In conclusion, we have now reached a point where HMOs are no longer an overlooked component of human milk, but considered to play a role in health promotion and disease prevention and treatment throughout the entire life span, from development of the fetus to frailty in the elderly (Fig. 2).

Disclosure Statement

The author declares no conflicts of interest related to this chapter.

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