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HUMAN MILK OLIGOSACCHARIDES: THE SECRET WEAPON FOR NEONATES AND INFANTS

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ABSTRACT

The human milk oligosaccharides (HMOS) range from 3 to 32 sugars in size, and differ greatly in composition from those of any other mammal. HMOs account for a notable quantity of human milk, similar to the quantity of total protein. The HMOs are "prebiotic" agents that selectively encourage the growth of beneficial (probiotic) organisms. In addition, the HMOS and their protein conjugates are recognized as pathogen-binding inhibitors that function as soluble "decoy" receptors for pathogens that have an affinity for binding to oligosaccharide receptors expressed on the infant's intestinal surface. These diverse milk glycans serve many functions, including protection and development ranging from selectively enriching gut bifidobacteria; prophylactically binding bacteria, viruses, and toxins; promoting the immune system; and enhancing intestinal epithelial barrier function. Mothers vary in the specific structures of HMOS in their milk as a result of genetic differences similar to blood group types. This variation in HMOS composition, unlike blood group types, does not create incompatability, so that all mothers may be considered "universal donors." Rather, the variation in HMOS composition among mothers is thought to promote human survival as pathogens differ in their affinity for binding to specific oligosaccharides. Protection by some forms of HMOS has been shown in relation to diarrhea caused by specific pathogens and HIV. The apparent differences in lactose and HMOS composition of preterm milk requires further investigation.

KEYWORDS: Human milk, oligosaccharides, immunity, pathogens.

INTRODUCTION

Human milk is a nutrient rich fluid with bioactive factors needed for infant health and development. Studies confirm that optimum nutrition and short and long term health benefits are attributed to the presence of functional ingredients such as proteins, lipids and oligosaccharides. Its composition varies with stages of lactation and between term and preterm infants.[1] While many studies of human milk composition have been conducted, components and variations in human milk are still being identified.

Human milk oligosaccharides (HMOs) are the third most solid rich component of human milk after lactose and fat. The constitution of HMOs varies with the duration of lactation and genetics of women. HMOs are present in a concentration of 20-24g/L in colostrum and then gradually declines in transitional milk and is least in mature milk (12-14 g/L).[2]

Structurally, HMOs are composed of 5 monosaccharides: L-fucose (Fuc), D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), and *N*-acetylneuraminic acid (Neu5Ac).[3] All HMOs are based on a lactose molecule (a disaccharide composed of a galactose molecule connected by a β1,4-glycosidic bond to a glucose molecule) so it is likely that HMO biosynthesis is an extension of lactose biosynthesis. All HMOs contain lactose (Gal-B1, 4-Glc) at the reducing end, which can be extended with lacto-N-biose I (Gal-b1, 3GlcNAc) or lactosamine (Gal-b1, 4-GlcNAc). Branching can be linear or branched through bonds β 1-3, β 1-6. The sequence can be further modified by the addition of Fuc and/or SA monosaccharides through α 1-2,3,4 and α 2-3,6 bonds due to the action of fucosyltransferases and sialyltransferases (figure 1).[4]

The three major HMO types present in human milk are **neutral**, **neutral N-containing** and **acid**. **Neutral (fucosylated) HMOs, representing about 35% to 50% of the total HMO content,** are neutral and contain fucose at the terminal position (e.g., 2′-fucosyllactose (2′-FL) and lactodifucopentaose). 42% to 55% of the total HMO content is the **Neutral N-containing (nonfucosylated) HMOs** which are neutral and contain *N*-acetylglucosamine at the terminal position (e.g., lacto-*N*-tetraose). Neutral HMO constitute more than 75% of the total HMOs present in human breast milk. **Acid (sialylated) HMOs, which represent 12% to 14% of the entire HMO** are acidic and contain sialic acid at the terminal position (e.g., 2′-sialyllactose).

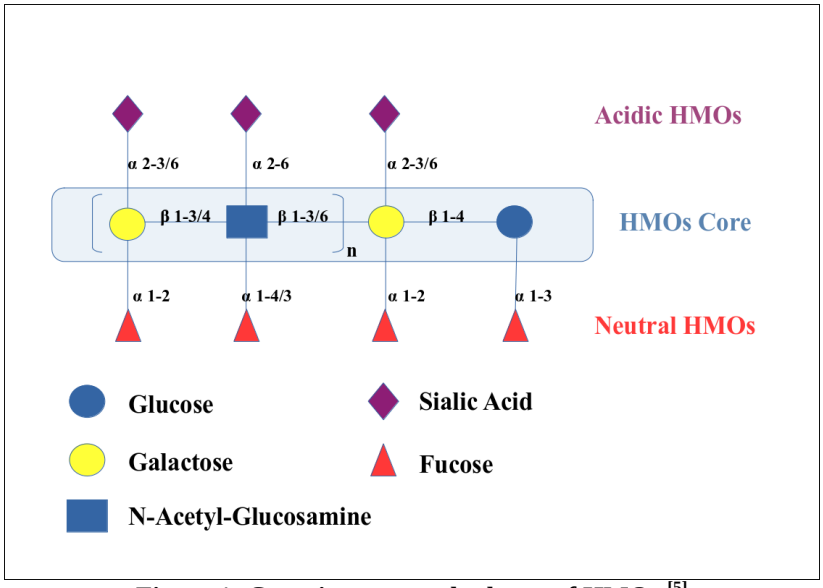

Figure 1: Generic structural scheme of HMOs.[5]

About 200 structurally different HMOs are known. In about 80% of all women, 2′-fucosyllactose is the most prevalent oligosaccharide with a approximate concentration of 2.5 g/L.[6] Other oligosacchardies include lacto-*N*-tetraose, lacto-*N*-neotetraose, and lacto-*N*-fucopentaos. HMO composition is influenced by maternal genetics including secretor and **Lewis blood group status.** Significant biological difference is evident in specific HMO structures such as 2′-fucosyllactose (2′-FL) and lacto-N-fucopentaose I and II (LNFP I and LNFP II) in mature human breast milk. The presence of α 1,2 fucosylated HMOs is dependent on the mother's genes encoding fucosyltransferase (FUT) enzymes,which is determined by the secretor (Se) status and Lewis (Le) blood groups.[7] HMO fucosylation is carried out by the 2 fucosyltransferases **FUT2** (**secretor gene)** and **FUT3 (Lewis gene).** Globally 30% women (Non secretor mothers) lack the functional FUT2 enzyme and produce milk devoid of α 1-2 fucosylated oligosaccharides like 2'fucosyllatose (2'FL) and lacto –N—fucopentaose (LNFP) I. Infants of these mothers are at a higher risk of diarrheal disease due the delayed colonization of bifidobacteria.[8]

HMO RECEPTORS

Many of the glycan motifs found on mammalian cells are also observed on microbes and food, including human milk. These similarities provide opportunities for host-microbe-HMO interactions. HMOs act as **soluble luring receptors** which prevent the attachment of pathogen to the epithelial cells.[4] Unbound pathogens are unable to attach to the cell surface and are excreted without causing disease. Many immunoreceptors also recognize the oligosaccharide structures of their glycoprotein ligands.

C-type lectins, **galectins**, **siglecs**, and **selectins are different** classes of lectins (glycan-binding proteins) that have different functions and ligand specificities.. Interestingly, different HMOs can bind to these different types of receptors, primarily expressed on cells of the immune system. For cellular function C-type lectins need calcium and include mannose binding lectin, selectin and dendritic cell (DC) specific intercellular adhesion molecule 3–grabbing non-integrin (DC-SIGN). DC-SIGN is expressed by gastrointestinal (GI) cells of infants and these GI cells are likely antigen presenting cells.[9] MUC1 is the major human milk glycoprotein that binds to the lectin domain of DC-SIGN and arrests pathogen interaction through the presence of Lewis x-type oligosaccharides.[9] The interaction of fucosylated ligands with DC-SIGN contributes to immune tolerance.[4,7]

Galectins play important roles for immune regulation and cell turnover. The carbohydrate recognition domain (CRD) of galectins is specific for β-galactosides. Galectins selectively bind to human milk glycans (HMG) and galectin HMG interaction play a crucial role in infant immunity.[10] Sigleces (Sialic acid–binding Ig like lectins) are sialic acid binding lectins mosly expressed on subsets of immune cells. Different leukocyte populations express siglecs which include sialoadhesin (siglec 1), CD 22 (siglec 2), myelin-associated glycoprotein etc. These are thought to promote cell to cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition.[11]

HMOs may bind to immune cells because of their structural similarity to selectin ligands.[12,13] Such bindings affect the populations and functions of immune cells. Sialyl-Lewis X glycotopes(sLeX), a glycoconjugate on the surface of the leukocytes, is involved in the process of leukocyte extravasation and mucosal infiltration. The E and P selectin, present on the surface of endothelial cells, recognize and interact with sLeX during inflammatory processes. HMO enzymatic modifications such as fucosylation and sialylation allow

binding to selectins.[14] Lewis X motif present in human milk can bind to DC–SIGN and subsequently prevent the capture and transfer of HIV1 to $CD4^{+}$T lymphocytes.[15]

FUNCTIONS OF HUMAN MILK OLIGOSACCHARIDES

HMOs act as Prebiotics

During the first 2-3 years of life the sequential development of infant gut microbiota take place that stars *in utero*. HMO is the energy source for *Bacteroids* and *Bifidobacterium* species that commonly colonize breastfed infants. In the large intestine, HMOs are fermented by *bifidobacteria* and produces acetic acid, which helps in the reduction of intestinal pH. It is bacteriostatic in nature i.e. inhibits the growth of pathogenic bacteria. In addition to acetic acid, fermentation products include butyric and propionic acid. The low pH in the gut also favors the growth of other beneficial strains of *bifidobacteria*. Evidence suggests that breast fed infants have a higher abundance of beneficial *bifidobacteria* compared with formula fed infants.[16,17] HMOs can indirectly increase short chain fatty acid (SCFA) production, and these elevated levels are mediated by bifidobacterial species. SCFAs are an important source of energy for enterocytes and colonocytes and are key molecules for maintaining intestinal health. Acetate, butyrate and propionate are dominant. Lactate and succinate, which are intermediate metabolites in SCFA production, are also present but are less studied. There is increasing evidence that SCFAs have a wider systemic effect because they act as signaling molecules and are involved in the regulation of many gene expression.[18] SCFAs have also been shown to play an dominant character in the activation and differentiation of immune cells and are associated with modulation of inflammatory and allergic diseases.[19]

HMOs modulate Intestinal Epithelial cell response

HMO affects innate immunity through the intestinal epithelial barrier. It reduce intestinal crypt cell proliferation, increase intestinal cell maturation and barrier function and may influence goblet cell function.[20]

The gastrointestinal system may be disorganized due to the inappropriate growth of glycocalyx on neonatal gut epithelium. Various studies indicated that 2′-FL and 3′-FL promote glycocalyx development in neonates in a structure dependent fashion which enhances gut barrier subsequently.[21]

Studies also imply that epithelial immune gene expression can be affected by the HMOs both directly and indirectly through the microbiota. 2-′FL, 6-′SL can arrest G2/M cell-cycle of two intestinal cell lines HT 29 and Caco-2Bbe. HMOs can reduce infection by inhibiting the growth of pathogens by modifying the gene expression of epithelial surface cells. Genes of Caco-2B gut cells mediate the adhesion between intestinal epithelial cells and *L. monocytogenes.* After pre-incubation with HMOs, the adhesion response were down regulated due to the activation of unfolded protein response and eIF2 signaling.[22] Studies suggest that HMOs can modulate the expression of tight junction protein, thereby decrease the permeability and enhance the barrier effect of the epithelium. Flow cytometric analysis and RT-PCR in different intestinal cell lines proved that HMOs can also alter the cell cycle dynamics by synchronizing regulator genes and mitogen–activated protein kinase signaling.[23] Expression of Muc2, the predominant form of mucin in the small intestine, which alleviates bacterial adhesion and permeability of the intestinal epithelium, have been shown to be upregulated by HMOs.[24] Disialyllacto –N-tetraose have been shown to give protection against necrotizing enterocolitis.[20]

Interestingly, the transformation from sialylation to fucosylation benefits the maturation of intestinal epithelium[25] and thereby HMOs can regulate intestinal epithelial cells through modulation of intestinal glycome.[26] The expression of sialyltransferases ST3Gal1, ST3Gal2, and ST3Gal4 are decreased in the presence of 3′-FL, leading to the reduction of α2-3-, α2-6-sialylation on Caco-2Bbe surface, which results in the reduced adhesion of *E. coli* by 50%.[27]

After fermented by *B.infantis* HMOs also impart indirect effects on intestinal epithelium. The conditioned media of *B. infantis* (BCM) enhanced expression of occludin and junctional adhesion molecule in either HT-29 or Caco-2Bbe, which can improve intestinal barrier function.[28,29] BCM also increased claudin-1 protein expression, by which the gut barrier was strengthened.[30] BCM might hamper IL-1b stimulation to protect Caco-2Bbe through NF-κB pathway as well.[29]

Antiadhesive antimicrobial properties of HMOs

For disease progression different viral, bacterial or protozoan pathogens need to adhere to mucosal surfaces to colonize or invade the host. HMOs can resist pathogens and provide a competitive advantage to non-pathogenic commensals and also reduce microbial infections by serving as antiadhesive antimicrobials.[7,8] Pathogen adhesion is often initiated by lectin–glycan interactions. *Escherichia coli* with S fimbriae as well as *Helicobacter pylori* bind to sialylated glycans and *Escherichia coli* with type 1 fimbriae bind to mannose-containing glycans.[31] Glycan-mediated attachment mechanisms are necessary for many viruses like noroviruses or rotaviruses, which are among the most common causes of severe diarrhea in infants and young children.[32] Some HMOs serve as **soluble decoy receptors** to prevent pathogen binding and reduce the risk of infections as they mimic the mucosal cell surface glycans.[33,34] Few HMOs showed significantly reduced binding and cytotoxicity of *E. histolytica* during *in vivo* assays.[35] A diverse comprehensive report of HMO as antiadhesive antimicrobials for *Campylobacter jejuni* infections are available, which is one of the most familiar causes of bacterial diarrhea, gastroenteritis and infant

mortality.[36] A significant scaling down of the pathogenic colonization occured in cultured epithelial cells after pre-incubation of Enteropathogenic *Escherichia coli* (EPEC) with mixed HMO components.[37] 2′-FL and LNFP I lower the rate of the adhesion of pathogens and decrease pathogenicity by binding to heat-labile enterotoxin type 1.[38] Lipopolysaccharide-mediated inflammation was instantly inhibited by 2'-FL during the process of enterotoxigenic *Escherichia coli* (ETEC) invading T84 and H4 intestinal epithelial cells. The uropathogenic *Escherichia coli* (UPEC) were also averted from adhering to epithelial cell monolayers in the presence of 15 mg/mL HMOs, which delay the p38 MAPK and p65 NF-κB s23ignaling pathways.[39] Attachment of *Streptococcus pneumonia* and *Haemophilus influenza* to human pharyngeal or buccal epithelial cells are lowered by HMO fraction of the breast milk.[40]

HMOs can prevent viral infections by mimicking receptor sites to block viruses from entering host cells[39] and subside virulence through γ-interferon and IL-10 expressions.[41] *In vivo* studies with human respiratory epithelial cell lines or peripheral blood mononuclear cells (PBMC) following respiratory syncytial virus (RSV) and influenza virus infection showed significant block of pathogen binding and reduced levels of IL6, IL8, MIP1 alpha, and TNF alpha after addition of 2′-FL, 6′-SL,3′-SL. In the same study 6′- SL was able to down regulate IL 10 and TNF alpha in RSV infected PBMC.[42,43]

Studies suggest that 2′-FL, 3′-SL, and 6′-SL have a notable antiviral activity against G1P[8] and G2P[4] rotavirus. 2′-FL significantly inhibited G1P[8] rotavirus infection, while a conjugate of 3′-SL and 6′-SL had the strongest ability to inhibit G2P[4] rotavirus infection.[44] However, HMOs cannot inhibit all kinds of rotavirus infections, such as neonatal rotavirus G10P, it has a dose-dependent enhancement in infectivity with the increased concentration of LNT and LnnT.[45] At physiological concentration, HMOs significantly lower the gp120 binding of Human Immunodeficiency Virus (HIV)-I virus to the dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) on human dendritic cells by 80%. In addition to preventing the gut virus, HMOs also can inhibit respiratory virus infections.[46] 2′-FL has been shown to reduce viral load of respiratory syncytial virus[42] and studies showed that the 2′-FL is able to enhance innate and adaptive immunity in influenza-specific murine model.[47]

HMOs as modulators of systemic immunity

As HMOs are structurally close to selectin ligands, it is assumed that they may bind directly to immune cells[12,13] Such binding can cause signaling which leads to changes in the growth and functions of immune cells. Studies with cord blood mononuclear cells suggest that human milk-derived oligosaccharides affect the cytokine production and activation of cord blood derived T cells *in vitro*.[12] It is assumed that in particular, acidic oligosaccharides may influence lymphocyte maturation in breast-fed newborns. Acidic HMOs are able to modulate cytokine production and activation of cord blood derived T cells *in vitro*. HMOs help in development of immune system in infants which leads to a more balanced Th1/Th2 response.[20]

Umbilical cord blood T cells exposed to sialylated HMO cause a rise in the number of CD3+/CD4+ and CD3+/CD8+ cells producing γ interferon, and CD3+/CD8+ cells producing interleukin-13 (IL-13). This is important because sialylated HMO is thought to affect lymphocyte maturation and promote the shift of T-cell responses towards more balanced Th1/Th2 cytokine production and low immunity. Some sialylated HMOs have been shown to contribute to the prevention of allergies as evidenced by lower cytokine production.[45,48–50]

Sialylated HMOs can bind sialic acid binding immunoglobulin-like lectins (Siglecs), notably Siglecs 5 and 9.[51] These receptors are found on neutrophils, monocytes and dendritic cells, and activation of these receptors after ligand binding leads to the apoptosis of neutrophils, limiting inflammation.[32] Sialylated HMOs present epitopes of selectin ligands, sialyl Lewis (x) and sialyl Lewis (a), and thus bind selectin inside the blood vessel, preventing selectin-mediated emigration of leukocytes. Sialylated HMOs can also reduce the formation of platelet-neutrophil complexes.[52]

Fucosylated HMOs are able to bind C-type lectin receptors named DC-SIGN receptors.[10] DC-SIGN is of particular interest because it is expressed by cells in the gastrointestinal tracts of infants. DC-SIGN receptors, present on the surface of both macrophages and dendritic cells, are involved in house dust mite allergy because their activation by different major house dust mite allergens induces the secretion of pro inflammatory cytokines.[53] Thus, HMOs could prevent asthma, allergic rhinitis and atopic dermatitis by binding DC-SIGN and preventing its activation by allergens.

CONCLUSION

HMOs are complex carbohydrates synthesized in breast gland which are abundant in human milk. Different kinds of HMOs directly or indirectly modulate the infant's physiological systems by regulating microbial composition, preventing pathogens adhesion and invasion, and regulating intestinal epithelial cell response. Also the rich diversity of HMO has the potential to modulate both innate and adaptive neonatal immunity. Supplementation of HMO to immune compromised population or at high risk for infection hopefully could benefit the overall health of neonates and infants.

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