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Human Milk Oligosaccharides an Inevitable Supplementfor Newborns.

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Abstract
Human milk is considered as golden standard for neonates in providing both short-term and long-term health benefits for infants such as lower risk of respiratory tract infections, diarrhea, otitis media, improving cognitive development, optimal growth rate with less obesity and T2DM(1). Human breast milk contains many macro & micro – nutrients. Besides water it constitutes solid components as Lactose 70g/L contributes the highest, Lipids 40 g/L, Human Milk Oligosaccharides (HMOs)5-15 g/L &Protein 8 g/L(1,2). HMOs are complex sugars that are present in breast milk, contributing to the third largest solid component with no nutritive function. Human milk contains diversity of components, more than 200 HMOs are identified and about 20 – 30 HMOs are quantified. Three main HMO categories based on the building blocks, (i) non-fucosylated neutral HMO which are the foundation for major HMO, most abundant among this is LNT. (ii) Fucosylated neutral HMOs – 2'FL is the most abundant in this category. (iii) Sialylated neutral HMOs – 3'FL &6'FL are the most abundant among this group. 2'FL, 3 SL, 6 SL are proven to be beneficial in improvement of memory, language, learning skills and attention. 2'FL, 3 SL, 6 SL, LNT & DFL play an important role in growth of beneficial gut bacteria & promoting gut barrier function, reduction of visceral pain, regulation of gut contractibility also it helps in decreasing infections by promoting adequate immune response (3,4). Apart from this many HMOs are shown to promote bone health and metabolic health. Preterm neonates specifically face health related risks owing to their immaturity and HMOs offer protective effect by shaping the Preterm immune system(5). Majority of the HMOs supplemented from breast milk reaches the lower gut unchanged only a small percentage of HMO is absorbed in the gut and reaches systemic circulation. They also have shown to have positive effects on Neuro development and cognitive development, help improve memory function at 24 months of life (6). Further studies in HMOs will be beneficial in improving the quality of life of a growingneonate.

Keywords: *Human Milk Oligosaccharides, Fucosylated, Sialylated HMO, FUT, Lewis antigen.*

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I. Introduction:

Breast milk is the ideal survival and natural food for infants, providing the required energy and nutrients that an infant need for the first few months of life. It also provides antibodies that helps the infant to protect against many infections. Composition of breast milk is unique with lactating women, apart from the essential nutrientsprovided breast milk has thousands of bioactive substances (1). The third largest solid component of breastmilk after lactose (55 - 70 g/L) and lipid (16 - 39 g/L) are the Human Milk Oligosaccharides (HMOs) (5 - 15 g/L). HMOs are one among the many bioactive substance in the breastmilk, which are non-digestible carbohydrates. The composition of HMOs that are present in human milk is 10-100-fold higher than which is present in cow's milk or any farmed animal milk. In 1920s the difference in stool microbiota of breastfed and formula feed infants were recognized for gut microbiota and associatedwith better health status of infants, the difference was understood to be due to the carbohydrate fraction. By 1950s most abundant

oligosaccharide were discovered in human milk this made oligosaccharides the most important bifidogenic factor in human milk [6]. The first HMO identified by Richard Khun in Germany, along with the bifidogenic factor as identified by Paul Gyorgy'sstudies in Heidelberg and later in Philadelphia USA [7]. HMOs can resist cold and heat and does not affect by freeze drying and Pasteurization. There were about 20 to 25 g/L of HMO in colostrum, and 10 to 15 g/L in maturemilk.

TYPES OF HMO

About three major HMO categories are present in breast milk,

- Fucosylated neutral HMO(35-50%)
- Sialylated acidic HMO(12-14%)
- Non-Fucosylated neutral HMO(42-55%)

The neutral HMOs contributes around 75% of the total HMOs in breast milk. As HMOs produced from mammary gland, the amount and composition vary between lactating mothers. The concentration being highest during early lactation and decreasing with time, studies also suggest HMO concentration is higher in a term than a preterm delivery[8].HMO content vary withLactationstages, Preterm or Term status of the mother, Secretor status or Lewis grouptype. Lewis antigen system is human blood group system based on 2 genes located on chromosome 19. Secretory gene is based on 19q13.3 and Lewis gene based on 19p13.3, both play a role in fucosylation of HMOs. All HMOs are secreted in mammary gland. Based on the genetic composition mothers secrete various HMOs.

Both FUT2 or secretor gene and FUT3 or Lewis gene are expressed in glandular epithelium. Secretor gene encoding FUT2 necessary for synthesis of 2'FL and other fucosylated HMO expressed in mammary glands. Acidic HMO (Sialylated) do not depend on secretor status. Mothers who lack secretor gene don't have 2'FL and α 1-2 fucosylated HMO, they are either Lewis +/- secrete 3'FL and Lacto-N-Fucopentanose (LNFP). LNFP are co-regulated with Se^+/Le^- and Se^- and Le +/- status. This specific influence explains the high concentration of 2'FL and other fucosylated HMOs in secretor milk (Se^+/Le^+). Infants with mothers who lack FUT3 have negative consequences as delayed colonization with *Bifidobacteria*[3,5].

HMOs are made of five monosaccharide molecules as Glucose (Glu), Galactose (Gal), N-ethylglucosamine (GlucNAc), Fucose (Fuc) and Sialic acid (SA). Almost all HMOs biosynthesis are based on extension of a disaccharide lactose molecule. HMOs Metabolism by gut microbes have shown that 1% is absorbed into circulation and rest is metabolized and excreted in urine and feces.

Se^+/Le^+ • 70% of the population. • FUT2 active, FUT3 active • Secrete all HMOs	**Se^+/Le^-** • 9% of the population. • FUT2 active, FUT3 inactive • Secrete Secrete 2′-FL, 3′-FL, LNFP-I, LNFP III
Se^-/Le^+ • 20% of the population. • Inactive FUT2, active FUT3 • Secrete 3'-FL, LNFP-II and LNFP III	**Se^-/Le^-** • 1% of the population. • Inactive FUT2, Inactive FUT3. • Secrete 3'-FL, LNFP-III and LNFP-V

2'-FL: 2-fucosyllactose; 3'-FL: 3-fucosyllactose; LNFP: Lacto-N-fucopentaose.

α 1-2 Fucosylated Transferase (FUT 2) – encoded by secretor gene and

α 1-3/4 Fucosylated Transferase (FUT 3) – encoded by Lewis gene.

HMOs now present in infant formulas with a mixture of Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) or inulin known to be bifidogenic. FOS are linear polymersoffructoseandGOSarelinerpolymersoflactoseatthereducingend.Infants fed with these HMOs along with FOS & GOS mix had gut flora similar to breast fed infants and had more predominance of *Bifidobacteria*and

less abundance of *Escherichia* and *Peptostreptococci*. Not only gut flora but also fecal concentration of propionate, butyrate and lactate were similar to breast fed infants [9].

BENEFITS OF HMO

A 、**Adhesive antimicrobial:** HMOs have shown to act as anti-viral and antimicrobial by acting as decoy receptors for pathogens and toxins. For a pathogen to infect a host it has to get attached to glycocalyx which is a carbohydrate layer coating epithelial cell surface and has glycans conjugated to proteins and lipids. HMOs act as prebiotic to symbiotic bacteria in the gut [6]. HMOs confers selective advantage to non-pathogenic beneficial bacteria than pathogenic as most of the seeker belong to bifidobacteriumwhich produces organic acids creating an acidic environment hindering the growth of pathogenic bacteria. Pathogenic bacteria that get attached to HMOs rather than the cell surface glycans pass through GItract harmlessly. Thus,HMOscompete with cell surface glycans and inhibit pathogen from causing infections. These have proven to be beneficial against various pathogenic bacteria's as a*Escherichia coli*, *Campylobacter jejunii*and viral infections as Rota virus or norovirus preventing from life-threatening gastroenteritis [2,5,6]. There are also few studies that have shown reducing fungal infection by *Candida albicans* and inhibiting the invasion of intestinalepithelium [10].

Glycocalyx is a major physical barrier preventing the invasion of toxin and binding with microorganism. HMOs helps in altering the glycosylation of epithelial cells. Corona et al proved that fucosylated HMOs enhances glycocalyx development in Caco2 cells 2'FL and 3'FL increases the thickness of absorbed albumin. 3'FL increased coverage area for albumin heparan cells and hyaluronic acid of Caco2 cells of glycocalyx [11]. All these functions reduce pathogen invasion and improves barrier function. Improvement in these functions in the colonic epithelial cells provide homeostasis of innateimmunity[12].

B 、Prebiotics: HMOs are carbon and energy sources mainly used by *Bifidobacteria*promoting growth in-turn intensify the production of lactic acid and Short Chain Fatty Acid (SCFA) to reduce the pH in gut making it suboptimalenvironment for pathogenic bacteria [3]. This in turn helps in development of immune system and multiple other function contributed by symbiotic probiotic functions contributed by Bifidobacterium [13].

C 、Immunomodulators: HMOs in modulating epithelial and intestinal cell responses.

Epithelial lining of small intestine and colon is an important barrier between gut lumen and circulatory system. Permeability of the layer depends on patency of tight junctions formed by the constant production and replacement of healthy cells on the intestinal crysts and villi [6]. Studies have found that HMOs increase the proliferation and differentiation of epithelial cells [12]. HMOs act by modulating the level of SCFA and maintain the epithelial barrier functions.

HMOs interact with lectins and TLRs in modulating the immune function

1. C – Type lectins: Expressed inAPC.
2. Galactins in T-Cells: Intestinal epithelialcells.
3. Selectins: Leukocytes or endothelialcells.
4. Siglecs in neutrophils, monocytes and dendriticcells.
5. TLRs in macrophages and dendriticcells.

Modulates various functions such as IgA transcytosis, T-Cell regulation, B-Cell function. Cytokine production activates NF-Kβ and intrinsic factor regulatory pathway to maintain immune system homeostasis. Imbalance in Th1/17 and Th2 phenotypes are noted at birth, whereas in neonatal period predominancy is towards Th2 phenotype promoting humoral immunity to confer protection for extracellular pathogens. Breast milk oligosaccharides hold a balance between Th1/17 and Th2 phenotypes and control the overexpression of inflammatory markers and regulate immune signaling [14]. Oligosaccharides also reduces the proliferation of mononuclear cells reducing the expression of IL2 and interferon γ [6]. *Bifidobacterium infantis*growth on epithelial cells shown to be beneficial in NEC[13].

D. Neurodevelopment: Sialylated HMOs provide sialic acid for production of ganglioside and glycoprotein needed for development of brain and cognition of brain. The presence of 2FL in early life improves cognition and memory [3]. Presence of sialylated and 2'FL have positive impact on brain neuronal development, memory and cognition. These HMO components in milk levels are shown to have effects at 24 months of age. As proven in a study conducted by Oliveros et al., pre-gestational diabetes, BMI status doesn't affect the composition of HMOs in mature milk that is necessary for neuronal and cognitivedevelopment [2].

II. Discussion

Carbohydrate in human milk is diverse with monosaccharide as glucose and galactose, disaccharide lactose, oligosaccharide, glycoprotein, glycolipids, glycopeptides. Berger et al., conducted a multicentered, randomized double blind interventional clinical trial from October 2012 to July 2015 in Italy with healthy full-term infants with 2.5 – 4.5 Kg, < 14 days of post-natal age were included for analysis. Healthy term infants received either infant formula without HMOs (control group) or same formula with two HMOs (test group). All randomized infants received the allocated formula for 12 months of age. Reference group of 38 exclusively breast-fed infants to 4 months, thereon introduction of solid foods was allowed. Stool samples were collected at 3 months and 12 months of age by parents and stored at home in-20^{O}C. Taxonomic composition in stool microbiota was analyzed by 16S rRNA gene sequencing. At 3 months infants on formula with 2 HMOs shifted stool microbiota composition and diversity towards breast-fed infants, the comparison with delivery mode also had similar trend. There was more abundance of *Bifidobacteria, Escherichia, Peptostreptococci,* and *Streptococcus* were modulated by HMOs. About 7 fecal community types were differentiated from the study population with predominance of *Bifidobacteria*in most breast-fed infants and significantly more in the test than in controlgroup (2).

Ferreira et al studied variation profile of HMOs throughout post-partum in a public health care center in Brazil during January 2017 and April 2019. Women of age 18 – 40 years, 25 – 38 weeks of gestation and free of any co-morbidities were included. Follow-up done at 5 visits as2-8 days (visit 1), 28– 50 days (visit 2), 88– 119 days(Visit3), 6 months (visit 4), 12 months (visit 5). Milk was collected and stored at – 80 ^{O}C and processed with HPLC-FL. 89.1% of women were found to be secretors. Component of HMOs isolated during visit 1, 2 & 3 were Lacto – N – Fucopentanose II (LNFP II), 3FL &2'FL respectively. HMOs produced were higher in early stage of lactation and decreased over time whereas it gets concentrated over the late period with a median of 16.66 mmol/L (12.5 g/L) at 2-8 days, 15.48 mmol/L (11.5 g/L) at 28 –50 days &16.79 mmol/L (11.3 g/L) at 88 – 119 days. Also, in this study they found the concentration of HMOs bound to sialic acid presented with a lower concentration at 3rdvisit than visit 1. In turn, HMOs bound with fucose were higher during visit 3 and lower invisit1.Inwomenwithpre-pregnancy3'FLwaspredominantinunderweight(BMI<18.5 Kg/m^{2}), Sialyl-Lacto-N-Tetrose c (LSTc) was predominant in normal (BMI18.5–24.9Kg/m^{2})and2FLrepresentedhigherconcentrationsforoverweight(BMI25–Kg/m^{2}) and obese (BMI >30 Kg/m^{2}) which showed pre-pregnancy weight and BMI directly correlated with Lacto-N-tetrose (LNnT) at visit 1. 2FL predominated in primigravida and LNnT in multigravida women. In secretor women 2FL was the major contributing HMOs during all three collection periods, however in non-secretor womenLNTatV1,LNFPIIatvisit2andvisit3wasmostabundant HMOs (15).

Parschat et al conducted a multicenter study to evaluate the tolerability, safety and effect on growth by doing a 16-weeksupplementation of 5 HMO – mix mimicking the natural concentration with 52% 2FL, 13% 3FL, 26% LNT, 4% 3'SL &5% 6'SL representing all 3 types of HMOs. The study was conducted in 12 sites across Germany, Spain & Italy during December 2018 & November 2020. Included healthy male and female infant full term (37 – 42 weeks of gestational age), singleton, <14 days of PNA at visit 1, birth weight of 2.5 – 4.5 kg and normal APGARs within 15 minutes of life. Control group was neonates on exclusive breast feeding and randomized intervention group receiving 5 HMO-mix or infant formula without HMO-mix. The 16-week intervention period was divided to 6 visits (Day0, day 14±3, day 28±3, day 56±3, day 84±3, day 112±3) followed by an8-week follow-up. 341 neonates were enrolled, 116 were in breast-fed group, 113 in 5HMO-mix group and 112 in infant formula group. Energy provided by HMO mix was 507 Kcal per 100g of the powder, infant formula 515 Kcal/100 g of powder. Weight gain was almost similar and no significant difference also compliance was found to be better. Theyhaveconcludedthatoveralladverseeventswasalmostsimilarbetweenthe3groups, which confirms the safety of HMOs. Total number of stools & watery stools in 5 HMO-mix was higher than infant formula group and lower in breast-fed group. Infants in HMO-mix group produced more softer stools fromV1-V4 compared to infant formula but not softer than breast-fed infants. No difference in regurgitation or flatulence in three groups. Vomiting was less in breast-fed group, crying was less in 5 HMO-mix than breastfed. Overall, this study concluded that the constituent of HMOs resembling breast milk is safe and well tolerated for use in healthy terminfants (16).

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