Bioactive Components of Human Milk
Including HMOs and Other Prebiotics
Lars Bode, PhD

Pediatric Nutrition
CONTINUING EDUCATION FOR CLINICIANS

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Faculty Presenter

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<table>
<thead>
<tr>
<th>Role</th>
<th>Disclosures</th>
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<tbody>
<tr>
<td>Speaker</td>
<td>Abbott Nutrition, Nestlé Nutrition, Nutricia/Danone, Medela</td>
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<td>Larsson-Rosenquist Foundation</td>
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Learning Objectives

Summarize ongoing research into the bioactive components of human milk

Describe the evidence for use of prebiotics in infant feeding
Human Breast Milk Composition

Introduction to Human Milk Oligosaccharides
The Composition of Human Breast Milk

Water

Carbohydrates
Lactose, HMOs

Lipids
Phospholipids, sphingolipids

Proteins

Immune Cells and Factors
Macrophages, cytokines, chemokines, immunoglobulins

Growth Factors and Hormones

HMO, human milk oligosaccharide.

Overview of Human Milk Oligosaccharides

- Nonnutritive carbohydrates found in breast milk\(^1\)
  - Indigestible and reach the small intestine and colon virtually intact

- Third most abundant bioactive component in human breast milk (after lipids and lactose)\(^1\)

- Unique to human breast milk\(^1,^2\)
  - Synthesis is highly energy intensive\(^1\)

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**Table: Macronutrient Concentrations in Human and Cow’s Milk**\(^2\)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Human</th>
<th>Cow</th>
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<tbody>
<tr>
<td>Protein (g/L)</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>Fat (g/L)</td>
<td>41</td>
<td>37</td>
</tr>
<tr>
<td>Lactose (g/L)</td>
<td>70</td>
<td>48</td>
</tr>
<tr>
<td>Oligosaccharides (g/L)</td>
<td>5-15</td>
<td>0.05</td>
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</table>

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HMO Structural Blueprint

- **5 basic building blocks** (monosaccharides) of HMOs\(^1,\)\(^2\):
  - glucose
  - galactose
  - N-acetylglucosamine
  - fucose
  - sialic acid

- **Over 150 HMOs** have been identified\(^1\)

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  - glucose
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\[ \begin{align*}
\beta1-4 \\
\alpha1-2 \\
2'-fucosyllactose (2'FL)
\end{align*} \]

- **Trisaccharide consisting of** glucose, galactose, and fucose
- **Most abundant HMO**, accounting for nearly 30% of all HMOs\(^1\)
- **Not all women synthesize**\(^1\)

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Maternal Variation in HMO Concentration

Absolute HMO Concentration

Relative HMO Abundance

CHILD, Canadian Healthy Infant Longitudinal Development study.

Genetic Drivers of Maternal Variation in HMO Production

- HMO composition in breast milk is determined in part by maternal genetics and the activity of genes in the Lewis antigen system
  - **Se (secretor) gene:** α1-2-fucosyltransferase (FUT2)
  - **Le (Lewis) gene:** α1-3/4-fucosyltransferase (FUT3)
- FUT2 is responsible for synthesis of 2′-FL and other α1-2-fucosylated HMOs
  - Women who do not encode a functional FUT2 enzyme cannot synthesize α1-2-fucosylated HMOs

<table>
<thead>
<tr>
<th>Lewis Gene (FUT3)</th>
<th>Secretor Gene (FUT2)</th>
<th>Phenotype</th>
<th>Associated HMOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>Lewis positive secretor</td>
<td>All HMOs</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Lewis positive non-secretor</td>
<td>LNT, LNFP II, LNFP III, LNDFH II</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>Lewis negative secretor</td>
<td>2′-FL, 3FL, LNFP I, LNFP II</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Lewis negative non-secretor</td>
<td>3FL, LNT, LNFP III, LNFP V</td>
</tr>
</tbody>
</table>

LNT, lacto-N-tetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose; FL, fucosyllactose.

HMO Composition of Mature Breast Milk Varies by Secretor Status

- Study of 96 milk samples from 32 mothers with preterm and term infants found significant differences in HMO composition based on genotype.

- Secretors had higher concentrations of 2’-FL and LNFP I, and lower concentrations of LNT, LNFP II, and LNDFH II.

LNFP, lacto-N-fucopentaose; LNT, lacto-N-tetraose; LNFDH, lacto-N-difucohexaose.

Geographic Variation in Secretor Status

Percentage of milk donors per country categorized as secretors

Maternal Diet Affects the HMO Composition of Breast Milk

According to a human crossover study of 14 lactating women (7-8 weeks postpartum and 7-9 weeks postpartum); bolded letters indicate non-secretors.

Maternal Diet Alters the Abundance of HMO-Bound Fucose and Sialic Acid in Breast Milk

HMO Composition of Breast Milk Changes Over Time

- The HMO composition of breast milk changes over the postpartum period.
- The concentration of most HMOs decreases whereas 2’-FL remains stable and 3FL increases.
- Changing composition of breast milk may reflect the changing needs of a growing infant.

Key Takeaways

- Human milk oligosaccharides represent a major bioactive component of human breast milk.

- Maternal variation in HMO composition is driven by a variety of factors, including genetics, geography, diet, and time.
Effects of HMOs
HMOs Promote Gut and Immune Health in Infants

A variety of HMO-dependent effects promote gut and immune health:

- **Prebiotic Effects**
  HMOs serve as metabolic substrates for beneficial bacteria within the gut

- **Antimicrobial Effects**
  Attachment of HMOs to epithelial receptors in the gut prevents microbial attachment

- **Transcriptional Effects**
  Signaling via HMOs alters the gene expression of intestinal epithelial cells

- **Immunomodulatory Effects**
  Cytokine production and inflammatory cell infiltration are modulated by HMOs

HMOs Stimulate the Growth of Beneficial Bacteria in the Infant Gut

• Undigested HMOs in the gut serve as metabolic substrates for beneficial bacteria, which form the basis of the infant microbiome\(^1\)

• HMOs promote the growth of bacteria that express sialidases and fucosidases, which can utilize HMOs, over other bacteria that cannot utilize HMOs as an energy source\(^1,2\)
  ▪ The *Bifidobacterium* genus is the dominant HMO-utilizing species in the gut of breastfed infants\(^1\)
  ▪ HMOs can also be utilized by some strains of *Bacteroides* and *Lactobacillus*\(^1\)

Microbial Diversity in the Gut Is Influenced by the HMO Composition of Breast Milk

- In an analysis of 412 milk and 406 infant fecal samples, the concentrations of several HMOs in maternal breast milk were significantly correlated with microbial community structures[1]

- In breast milk, most bacterial variants were negatively associated with sialylated HMOs and positively associated with fucosylated HMOs[2],[a]
  - Reversed for *Staphylococcus*

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HMOs Exhibit Unique Antimicrobial Properties Against Group B *Streptococcus*

- Approximately $10^5$ cfu *Streptococcus* were resuspended in tissue culture media with (solid) or without (dashed) HMOs (2 mg/mL)
  - Isolated from pooled human milk
- Group B *Streptococcus* growth was inhibited **10-fold** ($P < 0.05$)
- **Antimicrobial HMO:** LNT
- **Bacterial target:** Glycosyltransferase

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Inhibition of Bacterial Growth by HMOs

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Signaling by HMOs Regulates Immune Activity

- ~70% of immune cells are located in the gut\(^1\)

- HMO binding to cellular receptors can modulate immune-cell signaling and activity\(^2\)
  - **Galectins**: regulation of T-cell function
  - **Selectins**: leukocyte trafficking
  - **Integrins**: regulation of leukocyte interactions

- Acidic HMOs play a variety of immunomodulatory roles: \(^2\)
  - Downregulation of type 2 immune responses
  - Inhibition of T-cell proliferation

Health Implications: Necrotizing Enterocolitis

- Necrotizing enterocolitis (NEC) is a deadly intestinal disorder that occurs in upwards of 7% of preterm infants with very low birth weight (500–1500 g).\(^1\)
  - The mortality rate of NEC ranges from 10% to 50%, up to 100% for the most severe forms.
- The etiology of NEC is still unknown but is related to inflammation in the gut and failure of the intestinal epithelial barrier.\(^1\)
- Preterm infants who are breastfed are 6- to 10-times less likely to develop NEC compared with formula-fed infants.\(^1\)

Protection Against NEC May Be Associated With a Specific HMO

- In neonatal rats, HMOs (specifically, DSLNT) with 2 sialic acids were found to play a protective role against NEC\cite{1}

- In human infants, maternal breast milk DSLNT concentration was predictive of NEC development\cite{2}
  - DSLNT threshold level of 241 nmol/mL had a sensitivity and specificity of 0.9 for NEC

- Development of NEC may be related to microbiome composition\cite{2}
  - Infants with NEC had lower relative abundance of *Bifidobacterium longum* and higher relative abundance of *Enterobacter cloacae*

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{Concentration of DSLNT by NEC Status\cite{1}}
\end{figure}

HMOs Protect Against Some Infections:

**Diarrhea**
Higher relative abundance of fucosylated HMOs was associated with reduced incidence of diarrhea by 2 years.

**Respiratory Tract Infections & Gastroenteritis**
Higher LNFP II concentrations were associated with fewer cases of respiratory tract infections and gastroenteritis at 6 and 12 weeks.

**HIV**
Higher HMO concentration was associated with reduced risk for HIV transmission and mortality risk among HIV-exposed infants.

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*According to a systematic review of 6 original studies.[1]

LNFP, lacto-N-fucopentaose; HIV, human immunodeficiency virus.

...But May Enhance Infectivity of Other Pathogens

- Maternal breast milk HMO profile of infants with symptomatic rotavirus infection was distinct from that of rotavirus-negative and asymptomatic rotavirus-positive infants[a]

- Concentrations of LNT were most highly predictive of symptomatic rotavirus infection
  - Infectivity was also enhanced with the addition of 2'-FL and LNFP I in vitro

- LNT concentrations were positively correlated with Enterobacter/Klebsiella abundance and symptomatic infection

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[a] According to an analysis of 181 mother-infant pairs from Vellore, India.[1]

LNFP, lacto-N-fucopentaose.

Is HMO Supplementation a Substitute for Natural HMO Composition?

- In breast milk, HMOs exist as a complex mixture that changes with infant growth and development
  - Adding single oligosaccharides to infant formula is not equivalent to the mixture observed in breast milk

- Individual HMOs may have variable effects on infant health.
  - Lacto-n-neotetraose (LNnT): higher concentrations in breast milk are negatively associated with child height and weight between 3 months and 12 years of age[^1]
  - Disialyllacto-N-tetraose (DSLNT): lower concentrations in milk samples in NEC relative to controls[^2]

- Overall HMO composition and the ratios between them are likely to be more important than single oligosaccharides[^1]

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Emerging Research: HMO Composition Is Associated With Food Sensitivity Risk

- Overall HMO composition, but not individual HMOs, is associated with food sensitivity[1]

- LNFP III may be protective against cow’s milk allergy[2]
  - Infants with LNFP III concentrations <60 μM were 6.7-times more likely to develop cow’s milk allergy

Emerging Research: HMO Composition and Weight

- In a small cohort study (n=30 infants), HMO composition in breast milk was linked to excessive weight gain (weight-for-age z-score >2) over 6 months of age\cite{1}

- Individual HMOs may be associated with weight changes, but more research is needed
  - May have important implications for \textit{obesity risk}\cite{1} and \textit{malnutrition}
  - Considerations for formula supplementation

<table>
<thead>
<tr>
<th>Positive Associations Reported</th>
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<tbody>
<tr>
<td>DSLNT\cite{1,3}</td>
</tr>
<tr>
<td>2′-FL\cite{2}</td>
</tr>
<tr>
<td>3′-SL\cite{3}</td>
</tr>
<tr>
<td>3FL\cite{3}</td>
</tr>
<tr>
<td>6′-SL\cite{3}</td>
</tr>
<tr>
<td>DSLNH\cite{3}</td>
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</table>

<table>
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<tr>
<th>Negative Associations Reported</th>
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<tbody>
<tr>
<td>LNFP II\cite{1}</td>
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<table>
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<tr>
<th>Conflicting Associations Reported</th>
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<tbody>
<tr>
<td>LNNt\cite{1,2}</td>
</tr>
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</table>

DSLNT, disialyllacto-N-tetraose; 3FL, 3-fucosyllactose; 3′-SL, 3-sialyllactose, 6′-SL, 6-sialyllactose; DSLNH, disialyllacto-N-hexaose.

Emerging Research: Cognitive Development

Breastfeeding frequency and breast milk concentrations of 2′-FL at 1 month also correlated with infant cognitive development scores at 24 months.[1]

Key Takeaways

- HMOs support the development of the gut microbiome and immune system by serving as prebiotics, antimicrobials, and regulators of immune and epithelial cells.
- HMOs play a protective role against NEC and some intestinal infections.
- Emerging research suggests a role for HMOs in infant growth and development, including food sensitivities and cognitive function.
- Overall HMO composition may be more important for associated health benefits than individual HMOs.
Oligosaccharides in Infant Formula
HMO Supplementation in Infant Formula

- HMOs currently used to supplement infant formula: 2’-FL and LNNnT
- In a multicenter RCT, infants fed formula supplemented with 2’-FL (1 g/L) and LNNnT (0.5 g/L) experienced a shift in their fecal microbiome signature closer to that of EBF infants compared with those fed control formula[1]
- Compared with control formula, HMO supplementation was associated with less frequent respiratory infections, including bronchitis, and less antibiotic use[2]

RCT, randomized, controlled trial; EBF, exclusively breastfed.

Non-HMO Carbohydrate Prebiotics in Infant Formula

- Several non-HMO prebiotics are used to supplement commercial and clinical infant formula as well, including:
  - Galactooligosaccharides (GOS)
  - Fructooligosaccharides (FOS)
  - Inulin
  - Lactulose
  - Polydextrose

- The goal of addition of these prebiotics to infant formula is to produce a product that results in a **microbiome composition** closer to that of breastfed infants
  - May also serve as anti-adhesive microbials and possess anti-inflammatory properties

Non-HMO Prebiotics Support *Bifidobacterium* Growth

- In clinical trials, infant formula supplemented with inulin\(^1\) or GOS\(^2\) supported the growth of *Bifidobacterium* within the infant gut
  - High-fat infant formula supplemented with GOS increased the proportion of fecal *Bifidobacterium* to a level that was not statistically different from that of breastfed infants\(^2\)
- No difference in the incidence of gastrointestinal infections was observed,\(^1\) but prebiotic supplementation reduced the duration of infections\(^2\)

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Prebiotic Supplementation Provides Additional Health Benefits

Supplementation of infant formula with non-HMO prebiotics provides a variety of additional health benefits, including:

- Immune development and infection control[^1][^2]
- Healthy infant growth[^3]
- Prevention of allergy and atopic dermatitis[^4]

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Non-HMO and HMO Prebiotics Are Not Equal

• In an RCT, infants who were exclusively formula-fed were randomized to receive control formula containing GOS or formula supplemented with 2′-FL (0.2 or 1.0 g/mL)

• Infants fed 2′-FL-supplemented formula had lower inflammatory cytokine profiles compared with those fed control formula
  ▪ Similar to exclusively breastfed infants

Plasma Cytokine Concentrations in 6-Week-Old Infants

Control formula significantly different from all other treatments at P ≤.05 for all.

Discussion: Implications for Counseling New Parents

- Research on the effects of HMOs and other human milk bioactive components is still in the early stages
  - More research is needed to fully understand potential impact

- A detailed mechanistic understanding of HMO effects is required to guide formula product development

- There are potential opportunities for personalized early life nutrition
  - Not every HMO (either alone or in a blend) may be valuable or effective for every baby and situation
Key Takeaways

HMO (2’-FL and LNnT) and non-HMO prebiotics are added to infant formulas to support healthy gut development.

While non-HMO prebiotics provide a variety of health benefits, they are not equivalent to HMO prebiotics.