

## Efforts to emulate human milk oligosaccharides

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Research on human milk oligosaccharides (HMO) began with the characterisation of their chemical structures and is now focused on the elucidation of their biological roles. Previously, biological effects could only be investigated with fractions or structures isolated from breast milk; consequently, clinical observations were limited to comparisons between outcomes from breast-fed infants and their formula-fed counterparts. In some cases, it was inferred that the observed differences were caused by the presence of HMO in breast milk. Presently, analytical techniques allow for the fast analysis of milk samples, thus providing insights on the inherent variability of specimens. In addition, methods for the synthesis of HMO have provided single structures in sufficient quantities to perform clinical studies with oligosaccharide-supplemented formulae. Furthermore, studies have been conducted with non-mammalian oligosaccharides with the purpose of assessing the suitability of these structures to functionally emulate HMO. Taken together, these developments justify summarising current knowledge on HMO to further discussions on efforts to emulate human milk in regard to its oligosaccharide content. The present account summarises published data and intends to provide a historical context and to illustrate the state of the field.

### Paediatric Nutrition: Breast Milk: Oligosaccharides: Prebiotics

#### Introduction

Human milk oligosaccharides (HMO) vary in structure, size and composition and arise from the sequential addition of monosaccharides, such as L-fucose (Fuc), D-N-Acetylglucosamine, (GlcNAc), D-Galactose (Gal) and N-Acetyl-Neuraminic Acid (NeuAc) to lactose (Gal $\beta$ 1-4Glc). The smallest oligosaccharides are generated either when fucose is added to lactose, thus generating the trisaccharides termed fucosyllactoses (2'FL; Fuc $\alpha$ 1-2 Gal $\beta$ 1-4Glc and 3FL;Gal $\beta$ 1-4[Fuc $\alpha$ 1-3]Glc), or when the acidic monosaccharide NeuAc is added to lactose, generating the sialyllactoses (3'SL; NeuAc $\alpha$ 1-3Gal $\beta$ 1-4Glc and 6'SL; NeuAc $\alpha$ 1-6Gal $\beta$ 1-4Glc). The relatively high concentration of lactose in milk obscured until recently the presence of the oligosaccharide fraction, which was initially described with the name "gynolactose" and was later analysed and characterised by Grimmonprez and Montreuil<sup>1</sup>. In the same account these researchers hypothesised that lactose induces the synthesis of oligosaccharides; we now know that milk from other mammals may have high concentrations of lactose but contain a limited repertoire of oligosaccharides<sup>2,3,4,5</sup>. Oligosaccharides in milk from marsupials and monotremes are also different<sup>6,7</sup>. With time, it became apparent that milk possessed structures that were not found elsewhere in free, soluble forms. Researchers then began to isolate and characterise HMO to establish catalogues of possible glycoforms and elucidate metabolic pathways. Examples are two of the first comprehensive and systematic accounts of both analytical methodologies and chemical structures published by Kobata and coworkers<sup>8,9</sup>. During the last

two decades, explorations of both structure and function increased in number, depth and scope after the discovery of Lewis and Secretor blood group determinants in HMO<sup>10</sup>. Later on, structures were identified and characterised by biochemical and physical-chemical methods, in some cases corroborating the existence of molecular species<sup>11,12</sup>. As reagents such as polyclonal antibodies raised against immunogenic neo-glycoconjugates<sup>13,14</sup>, monoclonal antibodies raised against cell surface carbohydrates<sup>15</sup>, and lectins became available, it was discovered that HMO structures can also be found as components of glycolipids and glycoproteins<sup>16,17</sup>. Some of these cell surface oligosaccharides have structural motifs that are only expressed at certain stages of tissue development and occasionally reappear in tissues upon malignant transformation<sup>18</sup>. In fact, the use of a monoclonal antibody raised against pancreatic adenocarcinoma with specificity for an oligosaccharide epitope was proposed as a diagnostic tool for cancer<sup>15</sup>. Interestingly, from the discovery of the oligosaccharide fraction of breast milk to the discovery of antigenic determinants in particular structures, the study of HMO follows the development of the science of glycobiology, while emphasis on the nutritional roles of these carbohydrates has only been the focus of intense research in the last decade or so. Discussions on the roles of HMO in paediatric nutrition may be arbitrarily grouped into four areas of inquiry: 1) HMO as analogues of cell surface oligosaccharides used by pathogens and toxins for attachment to mucosa, thus acting as decoys that inhibit bacterial, viral or toxin attachment; 2) HMO as soluble fibres that could act as prebiotics promoting colonisation with beneficial bacteria, facilitating transit

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and preventing or controlling colonisation by pathogenic or putrefactive bacteria; 3) as sources of particular monosaccharides, and 4) HMO as substances that interact with the immune system and modulate its response. All of these areas represent actual or putative biological activities that are supported by varying amounts and quality of evidence. The main obstacle to discerning the biological roles of HMO has been the lack of availability of these structures in sufficient quantities and purity to clinically test hypotheses on their biological functions. Several clinical studies have been conducted to compare the physiology and clinical behaviour of breast-fed versus formula-fed infants and in some cases the results of these studies have been attributed to HMO; however, in these instances it has been impossible to ascribe the observed effects exclusively to oligosaccharides since other breast-milk components may elicit or boost particular responses. In the past decade, methods for the synthesis of HMO have yielded kilogram amounts of specific structures that in turn have been used to supplement infant formulae for clinical trials<sup>19</sup>. In the absence of HMO, several groups used non-human oligosaccharides to supplement formulae while seeking to emulate the physiological effects of human oligosaccharides. Two major classes of oligosaccharides have been assessed in clinical experiments in adults and infants and are now being used to supplement commercially available infant formulae: fructooligosaccharides (FOS) and galactooligosaccharides (GOS). FOS can be produced either by taking advantage of the reverse reaction of fructanases or sucrases<sup>20</sup> or through the enzymatic hydrolysis of inulin<sup>21</sup>, a polymer of fructose. Chemically, both types of FOS differ and commercial literature cites competitive advantages with each type. In terms of composition, FOS synthesised using glycosidases, frequently lack a reducing end and contain one glucose residue and two or more fructose moieties. These are well characterised, the structures have their own names<sup>22</sup>, and are sometimes termed short chain FOS (scFOS). The molecules generated from inulin have free anomeric carbons and generally contain only fructose. On the other hand galactooligosaccharides (GOS) are mostly produced from the catalysis of reverse hydrolysis using galactosidases<sup>23</sup>. Carbohydrates that cannot be hydrolysed and absorbed during their transit through the human gastrointestinal system but are hydrolysed and metabolised by beneficial bacteria residing in the human colon are considered to be “pre-biotics”. Both FOS and GOS fall within this definition and HMO behave partially in such a manner since some are also found intact in the faeces of breast-fed infants<sup>24</sup>. Therefore, both FOS and GOS have been studied for their abilities to emulate the physiological and biochemical effects of HMO. Although the rest of the present review is focused on experiences with HMO and breast milk, it is important to refer the reader to experiences with non-human oligosaccharides because, as stated above, formulae supplemented with these structures have already reached the market place.

### Human Milk and HMO

Since several HMO contain blood/tissue specific antigens, and lactating mothers belong to different blood/tissue antigen groups, milk samples are varied in their glycosylation. This is due to the fact that the HMO repertoire in a sample constitutes a phenotype of secondary gene products (oligosaccharides)

that result from the expression and activity of primary gene products (the enzymes that synthesise oligosaccharides). Erney *et al.* and Thurl *et al.* described HMO profiles that agree with the predicted variability of phenotypes according to the Lewis Blood Group and Secretor status of the donor<sup>25,26,27</sup>. More recently, Chaturvedi *et al.*<sup>28</sup> corroborated the findings of Enrney *et al.* in terms of HMO profile variability amongst samples and through lactation. Enrney and Prieto have assembled evidence of the existence of profiles that are not consistent with predictions based on known genotypes; the existence of these profiles was preliminary published in an abstract<sup>29</sup>. This evidence indicates that milk from different mothers may be qualitatively and quantitatively different in regard to its oligosaccharide content. This may also apply to other glycoconjugates, for example, glycoproteins that are decorated or remodelled by the same glycosyltransferases that synthesise HMO. Samples from Lewis positive mothers contain both the expected oligosaccharides resulting from the fucosyltransferase encoded by the Le gene and glycoproteins that react with monoclonal antibodies against antigens of the Lewis system. Similarly, transgenic animals expressing glycosyltransferases under a lactogenic promoter produce both neooligosaccharides and neoglycoconjugates<sup>30,31</sup>. The unavoidable conclusion is that breast-milk is different from one mother to the other. Some mothers synthesise fucosylated sugars such as Fuc $\alpha$ 1-2 Gal $\beta$ 1-4Glc, others do not, and these oligosaccharides can be present at concentrations up to 2 grams per litre according to our studies. If this oligosaccharide or, in general, Fuc $\alpha$ 1-2 Gal residues are important, some breast-fed infants are not being exposed to these structures through their mother's milk. With regard to glycosylation, breast-milk is heterogeneous.

### Clinical evidence that supports biological roles for HMO

Table 1 is a summary of selected results from reports that explored the biological roles of HMO. This table includes studies other than clinical experiences because its purpose is to illustrate prevalent research lines. Also, there is a limited amount of clinical studies that specifically shed light on HMO functions in the paediatric nutritional context. When studying the effects of HMO on breast-fed infants, two confounding factors arise: putative biological effects of HMO have also been ascribed to glycoproteins and glycolipids<sup>32,33</sup> and supra-molecular constituents of milk<sup>34,35</sup>, and glycoconjugates may share structural features with HMO. These two factors make it difficult to ascertain the particular roles of HMO in clinical studies comparing breast-fed versus formula-fed infants and, to our knowledge, only one clinical study with a formula supplemented with a human milk oligosaccharide has been reported in the literature<sup>19</sup>. Additional factors may confound interpretation of experiments aimed at probing the ability of particular oligosaccharides to function as prebiotics; one of these is the characterisation of species and varieties of components of the intestinal flora. A review of pertinent literature shows that increased faecal counts of *lactobacillus* and *bifidobacterium* species are considered to be beneficial outcomes of the ingestion of HMO or other oligosaccharides<sup>36</sup>. One publication suggests the importance of a particular species and variety of *bifidobacterium* and mentions that the oligosaccharide Lacto-N-neotetraose (Gal $\beta$ 1-4GlcNAc $\beta$ 1-3

**Table 1.** Selected studies that support particular roles for HMO

Type of Study	Key study characteristics	HMO tested	Main outcomes examined	Key findings	Ref.
<i>In vivo</i>	Ability of certain HMO to interfere with colonisation with <i>S. pneumoniae</i>	LNnT and sialylated forms	Lung colonisation of <i>S. pneumoniae</i> in rabbits. Pharyngeal colonisation of <i>S. pneumoniae</i> in rats	Decreased bacterial load in lungs (when administered intratracheally), prevented bacteraemia	50
<i>In vitro</i>	Ability of milk fractions (high or low molecular weight) to inhibit adhesion of <i>S. pneumoniae</i> and <i>H. influenzae</i>	A fraction of milk with Lacto-Neo series HMO	Adhesion of <i>S. pneumoniae</i> and <i>H. influenzae</i>	The oligosaccharide fraction of milk inhibited adherence of <i>S. pneumoniae</i> and the high molecular weight fraction inhibited adherence of <i>H. influenzae</i>	49
Clinical (Cohort)	Diarrhoea in breast-fed infant-mother pairs ( $n = 93$ ); mothers with differing Lewis blood groups	Fuc $\alpha$ 1-2 versus other Fuc $\alpha$ -R	Diarrhoea incidence in infants.	Milk with higher ratio of 2-linked fucosylated versus non-2-linked oligosaccharides conferred protection	41
<i>In vitro</i> and <i>In vivo</i>	Ability of 2'FL to inhibit adhesion of <i>Campylobacter jejuni</i> to target cells/tissues	2'FL	Adhesion of <i>Campylobacter jejuni</i> to cells in the presence of 2'FL; Ability of milk with 2'FL to inhibit colonisation in rat pups	2' FL inhibits <i>C. Jejuni</i> adhesion to WHAT??. Pups fed milk with 2'FL showed reduced colonisation	40
Clinical	( $n = 228$ ) Six-month and older infants; one group fed supplemented formula	LNnT	Colonisation of throat and nasopharynx with <i>S. pneumoniae</i> and <i>H influenzae</i>	Colonisation was not reduced, formula was well tolerated, trend to reduction of "inflamed" ears	19
Analysis of faeces	Presence of oligosaccharides in faeces	Several	Presence of oligosaccharides in faeces	Apparent different oligosaccharide metabolism in term versus preterm infants; Presence of oligosaccharides in faeces suggests they may act as fibres	44,45
<i>In vitro</i>	<i>In vitro</i> digestibility of HMO	HMO in general	HMO digestibility	Less than 5% of HMO are digested by human enzymes; HMO are available to act as prebiotics in the colon or as fibres	47
Analysis of faeces	Determine bifidobacteria and enterobacteria	Human milk	Bacterial counts of faeces from breast-fed and formula-fed infants	Enterobacteria are reduced in breast-fed infants. Bifidobacteria are dominant	46

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Gal $\beta$ 1-4Glc) is a particularly suitable prebiotic for such a microbe<sup>37</sup>. While this may be true for *in vitro* experiences there is no clinical evidence to support this assertion. Species and strains are sometimes confused because of the lack of standards to define them. More than ten years ago Dubey and Mistry<sup>38</sup> reported another confounding factor: species of bifidobacterium grow at different rates under the same conditions. These include degree of anaerobiosis, pH, particular nutrients and temperature. Conducting *in vitro* studies to determine the prebiotic effect of oligosaccharides is relatively easy and results of significant difference can be detected<sup>19</sup>. This is true when comparing bacterial responses to different oligosaccharides using either growth or selected metabolic or enzymatic activities as benchmarks. In contrast, similar experiences in the presence of relatively large concentrations of lactose (such as in milk) generate results that are difficult to interpret. When saccharide-supplemented infant formula is used to assess its prebiotic effect as a whole, other factors such as the concentration of lactulose (generated during the thermal process) become critical because its prebiotic effect may mask or enhance the effect of other saccharide structures<sup>39</sup>. In lactose-free formulations the concentration of sucrose becomes as important specially when assessing the effects of fructooligosaccharides. As indicated above, only one clinical study has been published in which a pure HMO was used to supplement infant formula. The oligosaccharide (LNnT) was present in formula at the average concentration in which it is found in breast milk (200 mg/l). Perhaps such a study should only be considered a proof of the concept that demonstrates the feasibility of conducting large-scale clinical studies with HMO-supplemented formulae. A detailed analysis of Table I reveals that a systematic effort has been conducted by Ruiz-Palacios and coworkers to probe the anti-pathogenic effects of fucosylated oligosaccharides, specifically those that contain the Fuc $\alpha$ 1-2 structural motif<sup>40,41</sup>. This group used CHO cells transfected with a human fucosyltransferase that catalyses the synthesis of Fuc $\alpha$ 1-2 residues. These cells express fucosylated glycoproteins in their cell surfaces which allowed them to compare the ability of strains of enteropathogenic *Campylobacter jejuni* to bind to these cells and to the wild type<sup>42</sup>. In addition they also took advantage of a transgenic animal model in which mice expressing the same fucosyltransferase express 2'FL in their milk<sup>30</sup>. The investigators inoculated newborn mouse pups with *C. jejuni* and compared the persistence of colonisation in pups fed by transgenic mothers and those fed by wild type mice. Pups fed with milk containing transgenically expressed Fuc $\alpha$ 1-2 oligosaccharides and glycoconjugates shed the pathogenic bacteria faster than pups fed with control milk. Furthermore, this group also conducted a cohort study to determine the effect of Fuc $\alpha$ 1-2 on diarrhoea<sup>41</sup>. Because some mothers do not synthesise this structure, they were able to ascertain that infants fed milk containing Fuc $\alpha$ 1-2 were less susceptible to diarrhoea. In this particular case there is a clear progression from *in vitro* to clinical relevance. Other studies have compared the ability of breast-milk to promote colonisation with bifidobacteria or lactobacillus, or to decrease counts of pathogenic bacteria, or have focused on quantifying HMO in faeces<sup>43,44,45</sup>. *Ex vivo* digestibility studies complement these observations<sup>46</sup>. The evidence suggests that the least controversial role for HMO is as primordial fibres and

prebiotics as elucidated by Coppa and coworkers<sup>47</sup>. Additional work has been conducted to determine if HMO can be used as nutritional anti-infectives<sup>48,49</sup>. In this regard, the work by Idänpään and coworkers was predicated on the ability of a particular structural feature of oligosaccharides to inhibit colonisation of epithelial cells of animals with *S. pneumoniae*. This work also evolved in demonstrations of efficacy in animal models and progress into the referred clinical trial<sup>19</sup>.

## Conclusions

HMO present unique problems for the assessment of their biological functions and the conducting of tests in the context of paediatric nutrition. The work of Ruiz-Palacios and coworkers suggests that infants fed with milk lacking or having low concentrations of 2'FL may be more susceptible to diarrhoea than their counterparts fed with human milk containing 2'FL<sup>40</sup>. This simple observation implies that emulating human milk and HMO is not as simple as previously thought. HMO may provide nutritional advantages to infants but may also have other applications. If it is true that fucosylated glycoconjugates that contain Fuc $\alpha$ 1-2 residues provide protection from diarrhoea in a paediatric nutritional context, then these saccharides could be excellent candidates to develop pharmaceutical applications for adults as well as infants. It is proposed here that it is no longer sufficient to reiterate that "more research is needed in the field of HMO"; now that these structures can be synthesised in large quantities it is feasible to pursue at least two classical paths of inquiry; 1) traditional *in vitro*, *in vivo* safety and pharmacokinetic studies<sup>50</sup> and 2) well-controlled clinical trials. The first approach may or may not lead to nutritional applications but may branch out as a specific type of pharmaceutical pool of drug candidates<sup>51</sup>. Recognition of subtleties and perhaps limitations imposed by nature on breast-milk and its components is the first step to understanding applications of HMO that extend beyond the realm of nutrition.

## Conflict of interest statement

PAP worked for Abbott Laboratories until March, 2007 and still holds stocks of this company in his retirement fund. Abbott Laboratories produces infant formula. RME and MT have no conflict of interest to declare. The article was co-written by all authors.

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