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Analysis and role of oligosaccharides in milk

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Milk is an important fluid in glycobiology because it contains a number of short carbohydrate chains either free or as glycoconjugates. These compounds as a class are the most abundant component and benefit the infant by developing and maintaining the infant's gut flora. New and emerging methods for oligosaccharide analysis have been developed to study milk. These methods allow for the rapid profiling of oligosaccharide mixtures with quantitation. With these tools, the role of oligosaccharide in milk is being understood. They further point to how oligosaccharide analysis can be performed, which until now has been very difficult and have lagged significantly those of other biopolymers. [BMB Reports 2012; 45(8): 442-451]

INTRODUCTION

Human milk is a unique fluid that is composed of lactose, lipids, free oligosaccharides and proteins, of which the free oligosaccharides are important constituents, at a concentration ranging from 5 to 23 g/L (1-3). Free oligosaccharides have the important role of establishing the gut flora of infants. Determining the role of these compounds has been led primarily by the analytical tools that made the rapid analysis and quantitation possible.

Free oligosaccharides in human milk can either be linear or branched, consisting of 3 to 14 monosaccharides (4, 5) (Fig. 1). It was initially believed that there are potentially thousands of structures. More recent analyses employing nanoflow liquid chromatography suggest perhaps a couple of hundred structures over five orders of magnitude for humans, with over 200 reported structures (4-7). For each human individual, there may be just over a hundred structures, based on over five orders of magnitude analysis. For humans nearly all of them originate from a lactose (Gal(β1-4)Glc) core further extended with N-acetyllactos-amine (LacNAc) repeats. These extended repeats can either be linked Gal(β1-3)GlcNAc (type I) or Gal(β1-4)GlcNAc (type II). Furthermore, both linear and branched structures can be decorated with fucose (Fuc) and/or N-acetylneuraminic acid (NeuAc). The NeuAc residues may be attached either with an (α2-3) or an (α2-6) linkage.

Variations in milk oligosaccharides are found between individuals and during the course of lactation. The major source of variation between individuals is associated with the person's Lewis blood group (8, 9). Fucose is a deoxyhexose saccharide that may be attached to HMO according to the expression of both the secretor gene (fucosyltransferase 2) and the lewis (fucosyltransferase 3) gene (10). Activity of the fucosyltransferases is regulated by genetic variation, and is thus inherited. Fucoses may be variably linked to a galactose. An α1-2 linked fucose is produced when fucosyltransferase 2 (FUT2) is active.. Alternately, fucose residues may be α1-4 linked when fucosyltransferase 3 (FUT3) is active (11, 12).

Although human milk oligosaccharides are the third most abundant component of human milk, they have no direct nutritional values. There are no glycosidases present in the infant gastrointestinal tract, and HMOs are therefore not digestible to the infant. Their abundances and their presence in every species of mammalian milk investigated thus far (13, 14), point to important functions in the development of the infant (15-19). HMOs have been found to be important for brain development. Studies with piglets suggest that sialic acids, an important component of HMOs, aid in brain development (20). Furthermore, HMOs act as decoys deflecting the binding of pathogenic bacteria (17). These anti-adhesive properties have been observed for several bacteria and viruses, including *Streptococcus pneumonia, Listeria monocytogenes, Vibrio cholera, Salmonella fyris,* HIV, enteropathogenic *Escherichia coli* and *Campilobacter jejuni* (21-23). It has also been observed that specific structures have distinct functions. *C. jejuni* is one of the major causes of diarrhea and was shown to adhere to 2'-fucosyllactosamine (23). Later, it was observed that the incidence of diarrhea in breastfed infants was directly related to the levels of 2'-fucosyllactosamine in their mother's breast milk (24).

More recently, HMOs have been associated with the growth of beneficial or commensal bacteria (25-27). In particular, several strains of bifidobacteria can grow well on HMO, however it was observed that some strains prefer fucosylated oligosaccharides, while others prefer non-fucosylated structures (28). Marcobal *et al.* reported recently that milk oligosaccharide con-
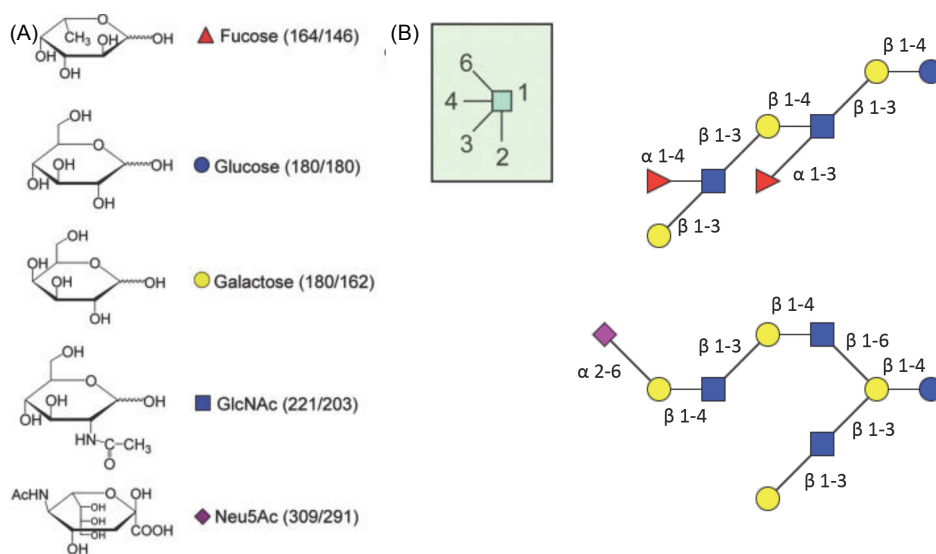
Fig. 1. Structural features of HMOs. Monosaccharide building blocks, together with their figural representation (A). Examples of HMO structures, which can be linear or branched and may be decorated with fucoses or sialic acids (NeuAc) (B).

sumption is not specific for bifidobacteria, but can also be observed for bacteriodes species (26, 29). In general, it is now established that human milk oligosaccharides have a strong influence on the composition of the gut microflora. It is proposed that a well-balanced intestinal microflora is important for the development of the infant's immune system (30), indicating that HMO play an important role in the infants well-being.

ANALYSIS OF HMOs

The key to the advancement in understanding the role of HMOs, has been the development of sensitive and quantitative methods for analysis. In this regard, a number of recent advancements have made this possible. The development of new mass spectrometry methods for ionization such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization allowed rapid determination of accurate mass as well as obtain structural information through tandem MS. The coupling of liquid chromatography with mass spectrometry allowed the profiling of oligosaccharide mixtures. Furthermore, nanoflow liquid chromatography yielded high sensitivity while microchip based devices yielded highly reproducible retention times.

Compositional profiling of HMOs
Complicated mixtures of milk oligosaccharides can be readily profiled by MALDI MS. Profiling of human milk oligosaccharides using MALDI-TOF-MS was first described by Stahl et al. (31), who were able to observe neutral oligosaccharides in positive mode as monosodium adducts as well as acidic oligosaccharides in both the positive and negative modes. It was noticed that desialylated fragments could be observed in the acidic fraction. This approach has been applied recently for the determination of lewis blood group by HMO fingerprinting. Following an auto-

mated oligosaccharide purification, HMO were analyzed using MALDI-TOF with 6-aza-2-thiothymine (ATT) as the matrix (9). Neutral oligosaccharides and sialyllactose could be observed as sodium- and potassium adducts in the positive mode, while other sialylated HMO were detected as deprotonated molecular ions in the negative mode. Using this method, 93.8% of the samples could be assigned the right blood group.

MALDI-FTICR-MS of oligosaccharides coupled mixture analysis with high mass accuracy and allowed the rapid determination of accurate compositions (32). Using 2,5-dihydroxybenzoic acid (DHB) as the ionizing matrix, neutral oligosaccharides were observed as sodiated adducts in the positive mode. The high-resolution analysis made the determination of composition relatively fast providing the identification of fucosylated and sialylated species. Furthermore, the high resolution of the FTICR-MS allowed application of deuterium labeled internal standards, which was shown to be simple and effective for rapid and accurate relative quantitation (28, 32, 33). Employing just this method, allowed the determination of the specific consumption of Bifido strains from infant gut (28).

Compound profiling of HMOs
The profiling of HMO structures requires the separation of compositions into individual components. Despite the advancements in liquid chromatography in normal phase (34, 35) and reverse phase (36-38) as well as in capillary electrophoresis (37, 38), there remains no single separation method sufficiently effective for separating HMO mixtures. The problem lies in the heterogeneity of the structures. While oligosaccharides are generally hydrophilic, the degree of hydrophilicity depends on the constituent monosaccharides. Those containing sialic acids tend to be slightly ionic compared to those containing "neutral" components.

Reverse phase (RP)-HPLC has long been used for oligosac-

charides. Native oligosaccharides are not retained on RP material, due to their hydrophilic properties, and therefore derivatization is required. A common derivatization method is permethylation, which was extensively used previously to stabilize and volatilize oligosaccharides for mass spectrometry analysis. Permethylation also aids ionization in modern instrumentation as it often stabilizes oligosaccharides during ionization thereby increasing sensitivity. However, it does require additional steps and partially methylated products can complicate analysis and decrease the effective dynamic range. Nonetheless, reverse phase is useful for the separation of permethylated oligosaccharides (39) employing standard reverse phase columns such as C_{18}. Unfortunately, RP of permethylated species lacks comprehensive separation of isomeric species.

Partial derivatization specifically at the reducing end can be used to make the compound amenable for reversed phase analysis. Chromophoric active tags such as 1-phenyl-3-methyl-5-pyrazolone (PMP), 2-aminopyridine (PA) and 2-aminobenzoic acid (2-AA) are commonly used for the analysis of HMO (40-43). These labels serve an additional purpose of providing a chromophore for spectrophotometric detection with HPLC. Stationary phases such as C_{18}, which do not normally retain or separate native oligosaccharides are also useful for chromato-

graphic separation.

Native oligosaccharides and HMOs are separated using normal phase with hydrophilic interaction liquid chromatography (HILIC). This method, which has already been applied extensively for the analysis of N- and O-glycans (44-46), was only recently applied to HMO (7). The oligosaccharides are labeled with 2- aminobenzamide (2-AB) using reductive amination to allow fluorescence detection, but separation occurs mostly through the hydrophilic oligosaccharide, and the elution-order is mainly influenced by the number of monosaccharide residues. Several sialylated isomers have been separated with this technique however extensive isomer separation of heterogeneous mixtures still creates problems.

Capillary Electrophoresis (CE) separation techniques have also been applied in the analysis of HMO (47, 48). CE with laser-induced fluorescence coupled to mass spectrometry (CE-LIF-MS) is effective for derivatized HMO. Labeling with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) introduces a fluorophore for the LIF detection, while simultaneously adding negative charge needed for the separation (49-51). However although good separation can be achieved using offline CE-LIF using very fast runs (around 9 minutes), both resolution and separation time have to be compromised when coupling CE to mass spectrometry (52).
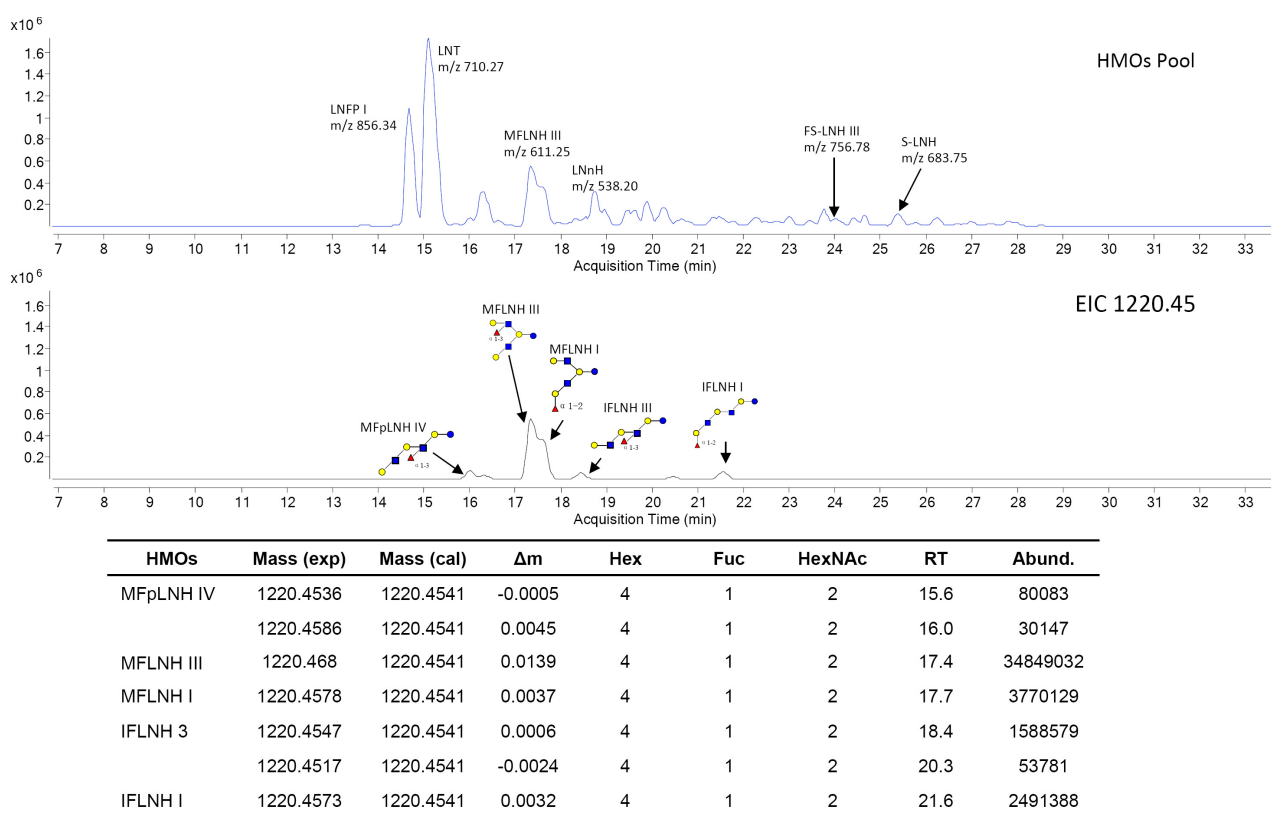

HMOs	Mass (exp)	Mass (cal)	Δm	Hex	Fuc	HexNAc	RT	Abund.
MFpLNH IV	1220.4536	1220.4541	-0.0005	4	1	2	15.6	80083
	1220.4586	1220.4541	0.0045	4	1	2	16.0	30147
MFLNH III	1220.468	1220.4541	0.0139	4	1	2	17.4	34849032
MFLNH I	1220.4578	1220.4541	0.0037	4	1	2	17.7	3770129
IFLNH 3	1220.4547	1220.4541	0.0006	4	1	2	18.4	1588579
	1220.4517	1220.4541	-0.0024	4	1	2	20.3	53781
IFLNH I	1220.4573	1220.4541	0.0032	4	1	2	21.6	2491388

Fig. 2. Isomeric HMOs can be separated on a porous graphitic carbon stationary phase. Base peak chromatogram with annotation of the highest abundant HMOs (above) and Extracted Ion chromatogram of mass 1220.45 with structural annotation (below).

Using micellar electrokinetic chromatography, native sialylated milk oligosaccharides may be separated, showing good isomer separation. The fixed negative charge of the sialic acid aids the separation. Native neutral HMOs are not effectively analyzed using such techniques.

Porous graphitized carbon is emerging as a highly versatile stationary phase for oligosaccharide separation. Our laboratory employs nanoflow chromatography with porous graphitized carbon (PCG) in a chip-based system coupled to a time-of-flight mass analyzer. The nLC-PGC-chip-TOF-MS combines the separation of graphitized carbon, which is excellent for separating isomers but still causes overlap of oligomers, with mass spectrometry, which is effective for identifying oligomers due to their differences in masses. The PGC is so effective at isomer separation that the reduction of the reducing end of the oligosaccharides is necessary because the α- and β- anomers are separated on the PGC stationary phase. Employed in the positive mode for the analysis of HMO (4, 5, 53), this method yielded extensive structural profiling and as many as 200 structures from five pooled milk samples. Furthermore, both neutral and sialylated compounds are readily separated in the same run (Fig. 2). When run using the same conditions, immediate identification is possible, using a library containing retention time, mass and fragmentation information (4, 5).

Structural characterization of HMOs

To determine function, comprehensive structural characterization is important. However, structural elucidation of HMO suffers the same problems as other oligosaccharides. Nuclear magnetic resonance yields the most extensive information regarding structure (e.g.(54, 55)), however, structural elucidation requires extensively purified compounds in significant amounts (typically micromoles). Mass spectrometry provides structural information on amounts that are six orders of magnitude less (picomoles), however complete structural analysis of oligosaccharides in not possible. Nonetheless, there have been significant efforts in developing methods that yield the most amount of structural information.

Traditionally, fragmentation of HMO is performed using collision induced dissociation (CID) on different types of instruments such as quadrupole ion trap (Q-IT)MS instrument (56, 57), quadrupole time-of-flight (QTOF) (4, 5), and Fourier transform ion cyclotron resonance (FTICR) MS instruments (58-60). Fragmentation behavior of milk oligosaccharides in both positive and negative mode has been reviewed extensively (61, 62). Cleavages of the glycosidic bond are most common. Cross-ring cleavages, which are necessary to obtain linkage information, are less abundant and often absent. Even when cross-ring cleavages are present, it is not possible to obtain the stereochemistry making it difficult to determine whether the residue is, for example, a glucose or a galactose. An important feature of the CID of oligosaccharides is the presence of re-arrangements in protonated species (63), which are the primary oligosaccharide ions produced in electrospray ioniza-

tion. Sodiated species, which are typically produced in MALDI, are not prone to internal migration.

Other activation methods such as electron capture/transfer dissociation methods have been applied to milk oligosaccharides (64). It was observed that electron transfer dissociation (ETD) resulted mainly in cross-ring cleavages, allowing unambiguous linkage-identification. However, the applications of these methods have been typically on simple standard compounds. It has yet to be determined whether these methods are sufficiently robust and reliable as CID for structural elucidation of oligosaccharides.

An effective method for determining structures employs the combination of tandem MS and glycosidase reaction. These methods use tandem MS to obtain connectivity and targeted enzymatic digestion to determine the residue as well as the linkages. With this method, the structures of over 70 neutral and sialylated milk oligosaccharides were elucidated. By characterizing the nanoLC retention times, the accurate mass, and the tandem MS, each structure can be identified in a complicated mixture (4, 5). In this way, rapid throughput characterization can be obtained on any HMO containing sample including feces and urine. In addition, this method also facilitates quantitation.

HMO quantitation

Along with structural identification, quantitation is another important capability to determine oligosaccharide function. While fluorescence and UV detection are traditionally regarded to be more robust for quantitation (40), they are not as suitable for oligosaccharides as they provide no structural information. Mass spectrometry based methods have been developed for quantitation but matrix effect and suppression in ionization tend to make this difficult. Oligosaccharides in mixtures tend to suppress each other resulting in different ionization efficiencies for specific components. For example, neutral oligosaccharides (those not containing sialic acids) will tend to suppress sialylated species in the positive mode while the reverse happens in the negative mode. However, separating the mixture into individual components produces responses to ionization and detection that are generally similar. In general, employing total ion counts in LC/MS is sufficient for most quantitative studies (e.g.(65, 66)).

For more accurate quantitation, isotopic labeling is desirable (28, 32, 33). Reduction of the aldehyde with sodium borodeuteride adds a deuterium to the resulting alditol. Mixtures can be analyzed by adding a standard mixture with deuterium to the sample, which has been reduced with sodium borohydride. With accurate mass instruments such as FTICR and TOF, quantitation is obtained by comparing the monoisotopic peaks of the hydrogenated and deuterated compounds, subtracting for ^{13}C abundances.

APPLICATIONS OF HMO ANALYSIS

Determination of genetic basis for human milk oligosaccharide production

The factors that determine the HMO profile of individuals has not been fully determined but it is generally agreed that there is a genetic basis for the structural diversity of each individual. The genetic relationship occurs between the Lewis blood group system and the structures of HMO found in an individuals' milk (8, 9). The genetic background of the Lewis blood group system has been thoroughly reviewed (12) and is depicted in Fig. 3. It is based on the linkage and position of the fucose residue. The fucose residues may be attached to HMO depending on the expression of both the Lewis gene (fucosyltransferase 3 – FUT3) and the secretor gene (fucosyltransferase 2 - FUT2). Fucose residues may be $\alpha1-2$ linked to galactose residues when Fut2 is active, while fucose residues may be $\alpha1-4$ linked when Fut3 is active (11, 12). In individuals where the expression of FUT2 occurs in epithelial cells, ($\alpha1-2$)fucose is secreted into fluids such as saliva, tear, and milk.

The most noticeable variations in structures and compositions observed by either MALDI-MS or LC-MS are due to the individual's secretor status (9, 67). Secretors tend to have similar profiles in their HMO due to the prominence of ($\alpha1-2$)Fucose structures in the profile, see Fig. 3. Some correlations are observed with the other Lewis blood group classifications, but these are not as rigorous (67). While there are a few studies relating the mothers' secretor status to infant health, there are a larger number of studies associating it to the individual's health. For example, it has been shown that secretors are less susceptible to infection by pathogenic bacteria. There have been, several reports that illustrate the beneficial effects of $\alpha1-2$ linked fucose on *E. coli* infection and the associated incidence of diarrhea in infants (22, 68, 69). Similarly, HIV binding to dendritic cells is blocked by the presence of milk oligosaccharides (21). Conversely, non-secretors are better protected to infection by norovirus (70, 71).

Selective consumption of milk oligosaccharides by the gut microflora

The interaction of HMO with the gut microbiota is an area of current and considerable interest. The interaction of a neonate with the microorganisms growing in the gastrointestinal tract is important for direct survival, but also health at the later stage of the infant's life. Human milk oligosaccharides largely influence the human microflora in the first weeks of a child's life (15), both by influencing binding of pathogenic organisms to the gut epithelial cells, but also by acting as a prebiotic (72, 73). Recent studies have focused on the consumption of oligosaccharides by gut bacteria. Several strains of bifidobacteria can grow well on HMO, however it was observed that some strains prefer fucosylated oligosaccharides, while others prefer non-fucosylated structures (28). Similarly, different galactoooligosaccharide (GOS) polymers were consumed differently by bifidobacteria strains (74). Marcobal *et al.* reported recently that milk oligosaccharide consumption is not specific for bifidobacteria, but can also be observed for bacteriodes species (29). While initial studies were performed using MALDI-FTICR-MS, more recent studies are performed using nLC-chip-TOF-MS. So far, spiking of the samples with a deuterated standard was performed in all cases to improve the quantitation of the HMO consumption, but this procedure does not only complicate the sample preparation procedure, but also requires specific data analysis protocols. New instrumentation (such as TOF-MS or QQQ-MS) and sample preparation methods should allow better quantitation without the need for internal standards. Using these techniques, it will be possible to screen bacterial HMO consumption much faster, and in a compound-specific manner (with linkage information), allowing better understanding of the processes that HMO are involved in.

Classification of primates based on milk oligosaccharide structures

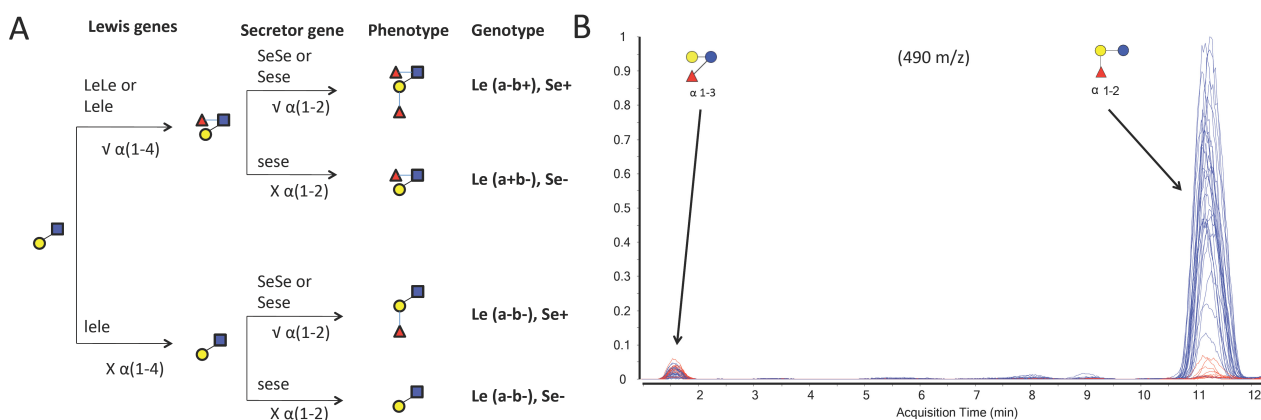Human milk oligosaccharides are the result of millions of years

Fig. 3. The HMO profiles are largely characterized by an individual's secretor status. Overview of the genetic background of the lewis and secretor genes (A). The levels of 2′-Fucosyllactose differ significantly between secretors and non-secretors (B).

of evolution (15, 16). Milk oligosaccharides of many mammals have been studied (13, 14, 75-81). However, studies from non-primate mammals are difficult to compare in quantitative terms because comprehensive profiling have not been performed. Nuclear magnetic resonance is the most common method for analysis. It is the only method that allows complete structural elucidation, however the sensitivity is severely limited being at over six orders of magnitude less sensitive than mass spectrometry. It therefore requires a large amount of material thereby prohibiting analysis for all but the most abundant components. Comprehensive profiling with quantitation is severely limited making comparisons between species difficult.

The new MS-based analytical techniques that allow quantitation and structural elucidation yield comprehensive oligosaccharide analysis making direct comparisons easier (Fig. 4). For example, bovine milk has been extensively studied and is generally low in fucose (<5%) but high in sialic acid (>60%). Other farm animals such as porcine and caprine are also highly sialylated but are closer to humans in terms of their fucosylation. The group that has been compared with extensive oligosaccharide profiles are the primates. Milk oligosaccharide analysis of several primates using nLC-PGC-chip-TOF-MS (14) shows that humans have a greater distribution of larger oligosaccharide compounds. Furthermore, chimpanzee, and rhesus exhibit higher fucosylation than humans, while Siamang and golden lion tamarin show very little fucosylation, on the same level as porcine and caprine. Conversely, Siamang has significantly higher sialylation, nearly equivalent to bovine. A further comparison of the milk oligosaccharides show it does not necessarily follow primate phylogeny. Instead, they can be clustered into the size of the social groups. Primates with large social groups tend to have

more similar oligosaccharide than those with small social groups. This is consistent with the notion that individuals in larger groups tend to be exposed to more pathogens that those who live solitary lifestyles. It also points to other more recent evolutionary processes, such as the prevalence of certain microbes in the gut flora, largely influence the structural characteristics of HMO (14).

CONCLUDING REMARKS

The ability to perform comprehensive profiles of milk oligosaccharides quantitatively will further allow greater understanding of the role of oligosaccharides in human milk. Milk is clearly an important excretion that deserve the kind of focus that has until now been reserved for other body fluids such as serum and plasma. The ability to identify and quantify large numbers of compounds has been key to this process. Overall, application of the novel analytical approaches will greatly enhance our knowledge on the specificities of bacterial consumption of milk oligosaccharides, and allow better understanding of the interaction between gut microflora (and thus the development of an infant's immune system) and HMO. Such knowledge should facilitate the development and clinical application of better infant formula, as well as personalized formula.

The study of milk has parallel the development of tools for the analysis of oligosaccharides particularly because milk is a highly glycosylated fluid. Indeed, milk has even encouraged the advancement of these tools. While oligosaccharide analysis remains a difficult task, the field is progressing rapidly so that the type of analysis performed on milk oligosaccharides can be performed on other biological fluids such as serum and plasma. The research further illustrates how effective analytical tools can en-
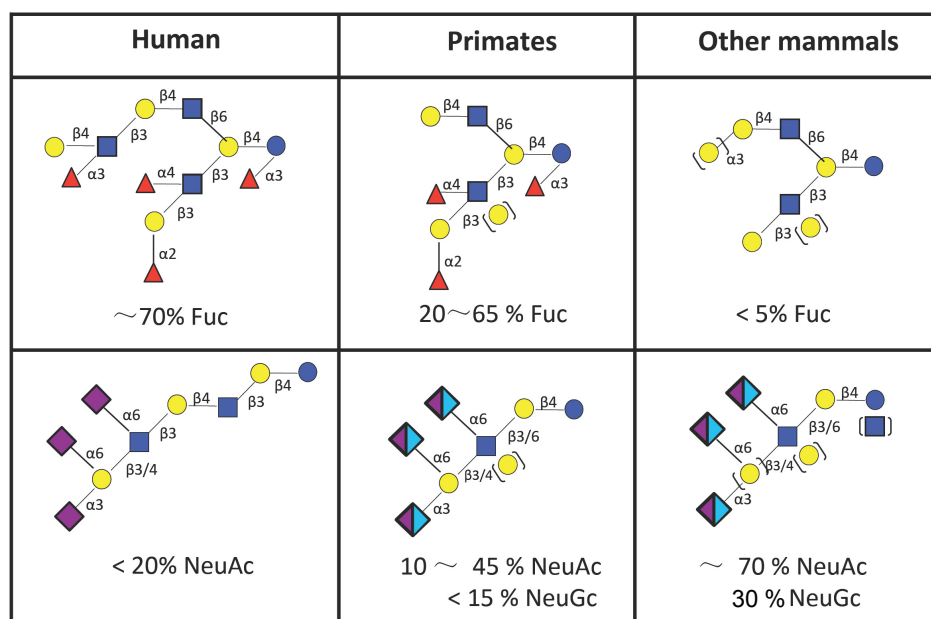

Human	Primates	Other mammals
~70% Fuc	20~65 % Fuc	< 5% Fuc
< 20% NeuAc	10 ~ 45 % NeuAc / < 15 % NeuGc	~ 70 % NeuAc / 30 %NeuGc

Fig. 4. The structural features of free milk oligosaccharides have changed during evolution. Human milk contains large amounts of fucose and highly branched structures, while lower levels of sialic acid are observed, relative to primates and other mammals.

able biology even allowing researchers to ask new questions.

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