Published in final edited form as:

J Agric Food Chem. 2011 April 27; 59(8): 4255–4263. doi:10.1021/jf104681p.

N-Linked Glycan Profiling of Mature Human Milk by High Performance Microfluidic Chip Liquid Chromatography Time-of-Flight Tandem Mass Spectrometry

David C. Dallas^{1,2}, William F. Martin¹, John S. Strum³, Angela M. Zivkovic^{1,2}, Jennifer T. Smilowitz^{1,2}, Mark A. Underwood^{2,4}, Michael Affolter⁵, Carlito B. Lebrilla^{2,3}, and J. Bruce German^{*,1,2,5}

¹Department of Food Science, University of California at Davis, One Shields Avenue, Davis, CA, 95616, USA ²Foods for Health Institute, University of California at Davis, One Shields Avenue, Davis, CA, 95616, USA ³Department of Chemistry, University of California at Davis, One Shields Avenue, Davis, CA, 95616, USA ⁴Department of Pediatrics, University of California Davis, 2315 Stockton Blvd., Sacramento, CA, 95817, USA ⁵Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

Abstract

N-linked glycans of skim human milk proteins were determined for three mothers. N-linked glycans are linked to immune defense, cell growth, and cell-cell adhesion, but their functions in human milk are undetermined. Protein-bound N-linked glycans were released with Peptidyl N-glycosidase F (PNGase F), enriched by graphitized carbon chromatography and analyzed with Chip-TOF MS. To be defined as N-glycans, compounds were required, in all three procedural replicates, to match, within 6 ppm, against a theoretical human N-glycan library, be at least two-fold higher in abundance in PNGase F-treated than in control samples. Fifty-two N-linked glycan compositions were identified and 24 were confirmed via tandem mass spectra analysis. Twenty-seven compositions have been found previously in human milk and 25 are novel compositions. By abundance, 84% of N-glycans were fucosylated and 47% were sialylated. The majority (70%) of total N-glycan abundance was comprised of N-glycans found in all three milk samples.

Keywords

Human milk; N-linked glycan; glycomics; prebiotics; glycobiology; glycoproteomics; glycoprotein; oligosaccharide; sialic acid; time-of-flight mass spectrometry; LC-MS

*Corresponding author (phone 530-752-1486; fax 530-752-4759; jbgerman@ucdavis.edu).

Supporting Information **Available:** The supporting information consists of a table of the mass and composition of all N-linked glycans identified in this research. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Safety: No special precautionary handling procedures were required for this work. No safety considerations were needed for this work other than normal laboratory safety precautions.

Introduction

Glycosylation, the most common post-translational modification, is the attachment of a saccharide chain to a protein at an asparagine (Asn) (called N-linked glycans) or at a threonine (Thr) or serine (Ser) residue (called O-linked glycans). The structural and compositional variety of these glycans, is immense (1). In human milk, N- and O-linked glycans are made up of monosaccharides including hexoses (mannose, glucose, galactose), N-acetylhexosamines (N-acetylgalactosamine, N-acetylglucosamine), fucose and sialic acid (N-acetylneuraminic acid (Neu5Ac)). Construction of these glycans occurs in a stepwise fashion in the endoplasmic reticulum and Golgi bodies of cells by the actions of a wide variety of glycosidases and glycosyl transferases, whose expression has been shown to be under partial hormonal control (2, 3). N-linked glycans (or N-glycans) are attached to the amide group of asparagine residues in well-known amino acid consensus sequences of Asn-Xxx-Thr/Ser and less commonly at Asn-Xxx-Cysteine (Cys) (where Xxx can be any amino acid besides Proline) (1, 4, 5). N-glycans have a common core made up of two N-acetylglucosamines (GlcNAc) and three mannoses (Man). Glycosylation affects protein structure, stability (6, 7) and proteolytic susceptibility and is essential in multiple processes including immune defense, cell growth, cell-cell adhesion (1, 8, 9), and pathogen binding. The structures and functions of these glycans in human milk and in the human infant have not been clearly defined and may differ significantly from those in other biological fluids and environments. Human milk protein glycosylations are of special interest because they are part of a food that has evolved with a dual role: nourishment and immune defense. Ninety percent of human milk oligosaccharides (HMO) survive intact in the feces of human infants, suggesting that they are, for the most part, not digestible by human infants (10, 11). Specific human milk N-glycans have been shown to have specific interactions with the normal human gut flora, including acting as decoys for pathogens (10, 12-14). In order to determine the biological effects and interactions of these glycans in the context of the neonate, their specific compositions and structures must be characterized.

Methods for total N-glycan release with PNGase F have been established, yet no study has yet assessed the N-glycan profile of human milk as a whole. Specific proteins from human milk, however, have been isolated and analyzed for N-glycans including bile salt-stimulated lipase, lactoferrin (M. Barboza et al., unpublished) and secretory immunoglobulin A (M. Barboza et al., unpublished) as well as some milk fat globule membrane proteins including clusterin, polymeric immunoglobulin receptor precursor, human milk fat globule EGF-factor 8 protein, and mucin 1 (15, 16). One study surveyed the Lewis type carbohydrate epitopes present on human milk proteins via SDS-PAGE of the protein mixture, but did not determine complete N-glycan compositions (17). From these studies, it is known that human milk N-glycans are highly complex with a wide variation in size, structure and abundance.

Determining the overall N-glycan profile of human milk protein is difficult due to analytical interference by the presence of the free HMO. As the third largest dry component of human milk, HMO overshadow the mass signals of the N-glycans released from the glycoproteins. In addition, distinguishing molecules as either N-glycan or HMO is difficult, as many have matching monosaccharide compositions, thus making them indistinguishable on a mass basis. Because of their large abundance, the removal of HMO from a sample of milk is

difficult and only partially successful with current methods. This means that an approach beyond mass matching is required to determine whether experimentally found compounds are HMO or N-glycans.

This study demonstrates a novel technique to examine the composition of the released N-glycans in human milk, and to distinguish these carbohydrates from the HMO present in the same sample. Each milk sample was analyzed twice, once with and once without PNGase F digestion and the two samples were compared to determine whether specific masses were due to enzyme release (and, thus, N-glycans) or if they were present in similar amounts in the sample before PNGase F incubation (and, thus, HMO).

This study employed nanoflow LC (nanoLC) with a graphitized carbon chip and orthogonal Time-of-Flight (TOF) MS for its ability to separate and detect heterogeneous mixtures with high resolution peak separation and high mass accuracy (routinely between 1-6 ppm mass error for complex carbohydrates) (18, 19). High repeatability in retention times, mass assignments, and high mass accuracy, makes this technique excellent for N-glycan profiling of human milk.

Materials and Methods

Human Milk Samples

Human milk samples were collected from 3 healthy, volunteer mothers who had given birth at term. Mature milk from donor A was collected at 3 months postpartum, while samples from donors B and C were collected at 4 months postpartum. Samples were kept at -80 °C until experimental preparation. The milk samples were collected as part of an ongoing lactation study. The UC Davis Institutional Review Board approved the study protocol and the subjects consented to the study prior to participation.

Milk Fat Fractionation

Milk samples were thawed on ice and centrifuged in 15 ml falcon tubes (VWR International) at $2,200 \times g$ for 15 min. The supernatant cream layer was removed with a metal spatula. Remaining skim milk infranates were centrifuged another 15 min and the residual cream layer was removed. The doubly-skimmed samples were used for the following steps.

Milk Protein Purification

This and all following sample preparation and instrumental steps were done in triplicate to produce three procedural replicates for each mother's milk sample. To remove HMO, 2 ml of -80 °C ethanol (Gold Shield, 200 proof) was added to each .5 ml doubly-skimmed milk sample, vortexed for 5 sec, centrifuged for 5 sec and then stored at -80 °C for 1 h to allow protein precipitation. Samples were then centrifuged at $16,000 \times g$ at 4° C for 15 min. The ethanol-water phase supernatant was removed by pipette, leaving the precipitated proteins.

Release of N-Linked Glycans from Human Milk by N-Glycosidase F Digestion

Two mg of precipitated protein from each sample were added to a 1.5 ml tube (Sarstedt). To this, 200 μ l of nanopure water (18.2 Ohm, E-Pure, Barnstead) and 200 μ l of 200 mM ammonium bicarbonate (Fisher Scientific), 10 mM dithiothreitol (Sigma Aldrich) were added. This mixture was vortexed and pipette mixed thoroughly until precipitated proteins were completely in solution. Sample solutions were then placed in 95 °C water for 2 min. Sample pH was checked and altered to between 7 and 8 with 1.0 M hydrogen chloride (typically required 15-20 μ l). Each sample was then divided into two equal parts (Figure 1). Two μ l of N-glycosidase F (PNGase F) (New England Biolabs, glycerol free) were added to one of each sample set, creating an experimental and control sample pair. All samples were then placed in a microwave reactor (CEM Corporation, North Carolina) for 10 min at 20 watts and 60 °C for microwave-assisted PNGase F N-glycan release, as described (20).

Deglycosylated Protein Removal

The remaining protein from each sample (treated and untreated) was removed, and released N-glycans were isolated via a second ethanol precipitation, performed according to the above-mentioned procedure, except that only 800 μ l of ethanol was applied to each sample and the N-glycan-containing ethanol supernatant was collected and dried completely by vacuum centrifugation (Genevac) at 40 °C (typically requiring 1 h).

N-Glycan Purification

All samples were then reconstituted in 0.55 ml nanopure water with 20 sec of vortexing. Solid phase extraction (SPE) was performed with 150 mg bed graphitized carbon cartridges (GCC) (Carbograph, Grace, Deerfield, IL). The extraction was performed with an automated Gilson GX-274 ASPEC liquid handling robot controlled by Trilution software (Middleton, WI). The extraction method was programmed as follows: Cartridges were first washed with 6 ml nanopure water, then conditioned with 6 ml 80% acetonitrile (ACN) (Fisher Scientific, HPLC grade), 0.1% trifluoroacetic acid (TFA) (Fisher, reagent grade) in water (v/v) and then washed again with 12 ml of nanopure water to remove the ACN. After conditioning, the N-glycan samples were loaded onto each cartridge and washed with 12 ml of nanopure water at a flow rate of approximately 1 ml/min to remove excess salts. The N-glycans were eluted with 4 ml of each of the following solutions in order at approximately 1 ml/min: 10% ACN in water (v/v), 20% ACN in water (v/v) and 40% ACN, 0.05% TFA in water (v/v). The 10%, 20% and 40% ACN fractions were used to elute sequentially neutral (10% and 20% ACN) and acidic N-glycans (20% and 40% ACN). Eluting solvents were collected in 5 ml freestanding sterile, silicon-sealed cryovial tubes (C-405, Phenix Research Products). Eluted fractions were immediately dried by centrifuge evaporation at 40 °C max temperature (typically requiring 3 hours) prior to mass spectral analysis.

Sample Preparation for LC-MS

All samples were rehydrated with 10 μ l of nanopure water and vortexed for 20 sec to resolubilize. Three μ l for each of the 10, 20 and 40% ACN fractions from each sample set were added to a 1.5 ml tube (Sarstedt) along with 11 μ l of nanopure water. Samples were vortexed and centrifuged briefly, and then added to autosampling vials.

HPLC-Chip/TOF-MS Analysis

All samples were analyzed with an established method (21) using an Agilent 6200 Series HPLC-Chip/TOF-MS system equipped with a microwell-plate autosampler, capillary sample loading pump, nanopump, HPLC-Chip interface, and the Agilent 6210 TOF LC/MS. The HPLC-chip used had a 40 nL enrichment column and a 43×0.075 mm² i.d. analytical column, both with 5 μ m porous graphitized carbon as stationary phase. Briefly, 2 μ L of sample was injected and sample loading was performed with 0.1% formic acid (FA) in 3.0% ACN in water v/v at 4 μ L/min delivered by the capillary pump. A gradient was set up as follows for the nanopump: at 0.3 μ L/min using (A) 0.1% FA in 3.0% ACN in water v/v and (B) 0.1% FA in 90% ACN in water v/v. A 45 min nanoLC gradient was run from 0 to 16% B, 2.5-20 min; 16 to 44% B, 20 to 30 min; 44 to 100% B, 30 to 35 min and held for 10 min at 100% B with an equilibration time of 20 min at 0% B. The drying gas was set at a flow rate of 4.0 L/min (2.0 L nitrogen and 2.0 L dry grade compressed air) and a temperature of 325 °C. Data acquisition was set to positive ionization mode within a mass range of m/z 400-3000 and a collection rate of 0.63 spectra/s. Continuous internal calibration was enabled using standard molecules of m/z 519.139 and m/z 1221.991 released from a tuning mix prior to the sample runs (ESI-TOF Tuning Mix G1969-85 000, Agilent Technologies, Santa Clara, CA). This system has been shown to consistently provide a mass measurement error of less than 3 ppm for N-glycans (21).

Chip-Q-TOF

After single MS analysis, N-glycan compositions were verified by rerunning all samples on a Chip-Quadrupole (Q)-TOF (Agilent) with the same sample concentrations and settings as for the Chip-TOF run, but with added tandem MS fragmentation. The same porous graphitized carbon (PGC) chip used for the Chip-TOF was used for the Chip-Q-TOF. Tandem MS was carried out with both automatic peak selection based on the three highest peaks in each MS scan and targeted selection based on identified N-glycan m/z values. Fragmentation energy was set at 1.8 V/100 Da with an offset of -2.4 V.

Processing Replicates

For each biological sample, the wet lab processing was replicated 3 times. Within each replicate, the biological sample was subject to both PNGase and non-PNGase treatment regimens yielding a pair of samples for MS analysis (for a total 6 per biological sample).

Interpretation of Mass Spectra

Initial data analysis was performed using a spectral data software analysis package (Mass Hunter Qualitative Analysis, Agilent). The program created separate compound chromatograms for each individual mass by combining sequentially eluting masses (using a function called Find by Molecular Feature). We restricted the retention time span for this compound search to between 7 and 37 min (to truncate the low amplitude wide tails of the time distribution of peaks). The m/z values were restricted to a range of 400-3000. Charge states were limited to a maximum of 3, since higher charges occur rarely in this context. Peaks less than 0.01% relative height or less than 100 counts were excluded. Target data type was set to small molecules (chromatographic). Extraction settings allowed for the

charge carrier to be hydrogen, sodium, potassium or ammonium. The isotope model used for the search was based on common organic molecule isotope patterns.

All compounds extracted were subsequently input to an in-house postgres relational database and compounds with masses matching those of a theoretical N-glycan library were identified. Algorithms were implemented as SQL queries and stored procedures which were written in plpgsql and plperl programming languages. The theoretical retrosynthetic N-glycan library was used because it represents a large number of possible permutations of N-glycan compositions, is believed to encompass all possible human N-glycans, and is not limited to what has been found experimentally. Possible carbohydrate compositions not part of this library were excluded. The possibility that N-glycans not present in this library exist in human milk will be explored in future work. The maximum error allowed between the experimental mass and the exact mass of the composition in the theoretical N-glycan library was 6 parts per million (ppm). Agilent's tests have shown that their Chip-TOF/Q-TOF equipment is sufficiently accurate so that an error threshold of 3 ppm is commonly effective. The distribution of mass errors for the selected N-glycans has a standard deviation of 3.8 ppm. A wider error threshold of 6 ppm was chosen since the triple confirmation of replicates chosen in our algorithm accentuates false negatives and greatly mitigates false positives. All compounds matching the theoretical N-glycan library were pooled, disregarding retention time.

Compounds with masses found in the retrosynthetic N-glycan list could not be unambiguously assigned as N-glycans, however, as a number of members of the retrosynthetic N-glycan library have compositional matches among the free HMO identified in literature. Therefore, a literature search was performed and a database was created of all known free HMO (108 entries) (22-30). This list was compared to the retrosynthetic library and it was determined that 36 or 10.8% of the 331 retrosynthetic N-glycan compositions matched compositions in the free HMO library.

A compound was considered as an N-glycan if the abundance ratios of PNGase F over non-PNGase F runs was at least 2:1 for all 3 processing replicate pairs. This step was necessary to eliminate carbohydrates that were HMO rather than released N-glycans. The data analysis is predicated on the assumption that the differences in compositional abundance within each pair are due to the presence of cleaved glycans occurring only in the PNGase samples.

A three step process was then adopted to verify that compounds matching the N-glycan library were N-glycan rather than HMOs.

First, compounds matching the retrosynthetic N-glycan list were further filtered by a two-fold abundance difference cut-off (based on combined compound peak volume) between the sample with and without PNGase F incubation. This step was necessary to eliminate carbohydrates that were HMO rather than released N-glycans. The data analysis is predicated on the assumption that the differences in compositional abundance within each pair are due to the presence of cleaved glycans occurring only in the PNGase samples. Compounds that appeared in the PNGase F fractions at abundances greater than 2-fold higher than the level in the non-PNGase F fractions were defined as candidate N-glycans

(Figure 1). Compounds with abundances in the PNGase F samples that were less than 2-fold higher than those found in the control samples could be HMOs or contaminants.

Second, to determine N-glycan abundances, for each control and experimental sample set, the abundance of the proposed N-glycans in the experimental sample were determined by subtracting the abundance of each particular mass in the control sample from the abundance of that mass in the PNGase F-treated sample. This determination is predicated on the assumption that the control samples contain no N-glycans. Abundances throughout this paper are based on “peak volume”, a column in the output generated by the “Find Compound by Molecular Feature” function of Agilent's Qualitative Analysis program.

Third, only compounds meeting the above two criteria for all three procedural replicates for a particular sample were accepted as N-glycans. By requiring the compound to meet the N-glycan criteria in all three procedural replicates allows for the removal of many false positives. For those compounds that pass the test for all procedural replicates, the abundance is calculated as the average of the abundances amongst the replicates.

Tandem MS analysis was performed manually on proposed N-glycan peaks with Chip-Q-TOF data. All tandem mass spectra were charge deconvoluted prior to analysis Mass Hunter Qualitative Analysis. Deconvolution settings included only two filters: a maximum assigned charge of 3 and a common organic molecule isotope model.

Results and Discussion

Identification and Clarification of Compounds Matching Both Theoretical N-glycan and HMO Libraries

From all three sample data sets combined, 72 compounds with masses matching the retrosynthetic N-glycan library were identified (data not shown). Of the compounds matching the N-glycan library, 22 (31%) were found to also match the HMO library mass list. These 22 compounds were assessed for N-glycan type abundance ratios in samples with and without PNGase F. Five (23%) of these matches were found in abundances at least two-fold higher in the PNGase F samples compared with the control samples and, thus, were defined as N-glycans in this study (Table S1). Seventeen (77%) were found at abundance levels less than two-fold higher in the PNGase F samples compared to the control samples and, thus, were defined as free HMO (data not shown).

Verification of Non-Ambiguous N-Glycans by Comparison of Samples With and Without PNGase F

Fifty of the compounds matching the retrosynthetic list did not match HMO compositions from literature. Lack of presence in the known HMO library did not, however, confirm that these compounds were not HMO due to the potential for unreported HMO compositions. Forty-seven of the compounds matching the retrosynthetic N-glycan library mass list but not the HMO library mass list were verified by abundance ratios to be N-glycans in the sample, whereas three were found to not be N-glycans (data not shown). These 3 compounds could be either very low abundance N-glycans, unreported HMO or non-carbohydrate contaminating compounds.

Confirmed Human Milk N-glycans

After these filtering steps, 52 N-glycans were confirmed in the three milk samples. These 52 N-glycans were compared with a library of known human milk N-glycans created from literature and it was determined that of this total, only 27 have been previously reported in literature. Thus, this study reports 25 N-glycan compositions in human milk that have not been previously published. A list of confirmed N-glycans identified in this study is provided in the Supporting Information (Table S1). The top 10 most abundant N-glycans are reported in Table 1 in order of abundance in the three samples. None of the top 10 N-glycans share a monosaccharide composition with a known HMO. Twenty-four of the proposed 52 N-glycans identified were compositionally confirmed via tandem mass spectrometry by Chip-Q-TOF. The 24 N-glycans confirmed by tandem made up 88% of the total N-glycan abundance in the three samples. Of the 24 N-glycans confirmed by tandem mass spectrometry, 9 were N-glycans not previously identified in human milk.

The 52 N-glycans identified in the three samples ranged in size from 7 residues (just two N-acetylhexosamine (HexNAc) added to the 3 Hexose (Hex) 2 HexNAc N-glycan core) to 18 residues, and in mass from 1234.43 (5-Hex 2-HexNAc) to 3246.18 (7-Hex 4-Fucose (Fuc) 6-HexNAc 1-N-acetylneuraminic acid (Neu5Ac)) Daltons. The ranges of monosaccharides in the N-glycans identified ranged from 3-9 for Hex, 2-6 for HexNAc, 0-4 for Fuc and 0-2 for Neu5Ac (also called Sialic Acid).

Fucosylation and Sialylation

Of the 52 N-glycan compositions identified in the combined data from all three samples, 34 (65%) were fucosylated, 20 (38%) were sialylated and 13 (25%) were both sialylated and fucosylated.

Abundance of sialylated, fucosylated and fucosylated-sialylated N-glycans were calculated by determining the ratio of the sum of peak volumes of a specific N-glycan group over the sum of peak volumes of all N-glycans identified (peak volume is a measure of peak abundance). All abundance values are presented as a percentage of the overall abundance. Of the total N-glycan abundance, 84% was fucosylated N-glycans, 47% was sialylated N-glycans and 38% were N-glycans carrying both fucosyl and sialic acid residues. Of the top 10 most abundant N-glycans identified, 8 were fucosylated and 4 were sialylated (Table 1). Of all the fucosylated N-glycan compositions identified, 16 (47%) had one, 8 (23%) had two, and 7 (21%) had three, 3 (9%) had 4 fucosyl residues. By abundance, 36% of the fucosylated N-glycans had one Fuc, 32% had two, 14% had three and 2% had four. Of the sialylated N-glycan compositions found, 17 (85%) had one and 3 (15%) had two Neu5Ac acid residues (by count). Of the total sialylated N-glycan abundance, 44% was made up of mono-sialylated and 3% was made up di-sialylated N-glycans.

Individual N-glycan Chromatograms

A software package (MassHunter Qualitative Analysis, Agilent) performed advanced peak finding for generation of extracted compound chromatograms (ECC) for all samples. Each ECC peak is a composite of the extracted ion chromatograms (EIC) that belong to a given compound and is a result of multiple isotopes, charge carriers and charge states. We used

ECC instead of EIC because it allows for increased peak intensity and simplified data analysis. The ECC of a representative sample (donor A) were labeled with structures for the most abundant N-glycans (Figure 2). Each ECC peak represents a single N-glycan molecule.

The compositions of the glycans represented here have been verified to be correct, however, the specific structures have not yet been determined. Therefore, the structures depicted are only a visual placeholder for compositional assignment, not the verified structure. The structures presented were based solely on composition and potential structural data on human N-glycans from literature (31, 32). All glycan structures depicted with arrows pointing to multiple ECC peaks have more than a single structural isomer in the sample; models shown represent only one possible structure for each N-glycan composition.

As can be seen from the ECC Figure 2, acidic N-glycans (those carrying sialic acid) eluted later than the neutral N-glycans (starting ~6 min after the first N-glycans eluted). These results are consistent with previous data suggesting that sialylated oligosaccharides have a higher affinity for graphitized carbon than neutral oligosaccharides (18, 21, 33).

Extracted compound chromatograms of one particular N-glycan composition, 5 Hex 4 HexNAc 1 Fuc (894.33 m/z) are shown in Figure 3A to illustrate the number of isomers that may be found for a single composition. For this composition, 6 peaks eluting at different retention times were found, suggesting that at least 6 structural isomers of 5 Hex 4 HexNAc 1 Fuc exist in the sample (it is possible, however, that some peaks may not be isomers, but rather alpha or beta anomers (21)). Structural isomers can differ by order or linkage position. The N-glycan mass associated with 5 Hex 4 HexNAc 1 Fuc is expected to have many positional isomers, some of which have been separated and partially elucidated with tandem MS for this study. This N-glycan composition could have 6 different isomers in the sample, as the previously published retrosynthetic N-glycan library (34) predicts 18 potential isomers for this complex type glycan. Assessment of putative isomers was performed through analysis of tandem MS fragmentation spectra (Figure 3B-D). Two of the structures were shown to have Fuc attached to an antenna according to trisaccharide b-ion with m/z 511.29 (Figure 3B and 3D) (35). Each of the three proposed structures here demonstrate the types of biologically plausible N-glycans that correspond with the tandem MS data, particularly the unique terminal residue losses as shown by the mass difference between y-ions for the loss of two sequential Hex in Figure 3B, the loss of terminal monosaccharide HexNAc in Figure 3C, and the loss of the disaccharide 1 Hex 1 HexNAc in Figure 3D.

Variations in Human Milk N-Glycans of Different Individuals

Examination of the three mother's milk samples here is not meant to determine specific significant differences between different mothers, but rather to show a preliminary range of the biological diversity of N-glycosylation. Future studies will further address the issue of inter-mother comparison in more detail. The number of N-glycan compositions identified in the individual donor milks are shown in Figure 4 and ranged from 22 (donor C) to 46 (donor B). These counts reflect only the number of compositions that were above the limit of detection of our experimental method (in the low femtomoles range (25)); many additional N-glycan compositions unreported here may exist in each sample. Abundances of particular

N-glycan compositions were computed as the average among processing replicates of abundance of mass matches.

The data were mined for overlap of specific N-glycan compositions among the three samples. Fourteen N-glycan compositions were detectable in all three sample sets (and in all three procedural replicates within each sample set). Those fourteen N-glycans made up 67%, 65% and 88% of the total N-glycan abundances in samples from donors A, B, and C, respectively. Twenty-eight N-glycans were detected in only two of the three samples. Ten N-glycans were identified in only one sample out of the three (4, 5 and 1 N-glycan compositions for donor samples A, B and C, respectively). The percent abundances of all N-glycans unique to each donor sample were <1%, 1% and <1% for donors A, B, and C, respectively. Therefore, the vast majority of N-glycan abundance in each sample was made up of N-glycans found in all three donor milks. Only a very small percentage of the overall N-glycan abundances were made up of N-glycans unique to a specific donor sample. These results must be understood in the context that other glycan compositions could be present, yet undetected due to the method's limit of detection.

Percentage of fucosylation by numbers of fucosylated compositions identified over the total N-glycans identified for that sample ranged from 61% (donor B) to 73% (donor C). The percentage of the total sample N-glycan abundance made up of fucosylated N-glycans ranged from 83% (donor A) to 87% (donor C). By compositional count, sialylation ranged from 35% (donor A) to 54% (donor C). By abundance, sialylated N-glycans ranged from 42% (donor A) to 66% (donor C) of the total N-glycan abundance. By compositional count, the percentage of N-glycans containing both Neu5Ac and Fuc ranged from 15% (donor C) to 19% (donor B). By abundance, the percentage of N-glycans with both Fuc and Neu5Ac ranged from 8% (donor C) to 18% (donor B). In all donors, the percentage of fucosylated N-glycans by abundance was higher than that of sialylated N-glycans (data not shown).

Fucose Biology

Compositionally, 65% of N-glycans identified contained at least one Fuc and by abundance, 84% of all N-glycans found in this study were fucosylated. This prevalence of Fuc on human milk N-glycans may have relevance to the developing infant immune system. HMOs with α 1,2 fucosylated epitopes have been shown to prevent adhesion of pathogens including Noroviruses (NV) (36, 37) and gram-negative bacteria (specifically *Campylobacter jejuni*) (38) to intestinal cells. Free milk glycans have been proposed to competitively inhibit the first step of pathogenesis by binding to bacteria and preventing their binding to their usual target—intestinal cell-surface glycans (39). Indeed, studies have shown that infants fed breast milk with high levels of α 1,2 fucosylated HMOs are less likely to acquire *E. coli* infections, campylobacter diarrhea and NV diarrhea (40, 41). This inhibition of pathogen binding is essential immune protection provided to the infant by breast milk (39).

The glycans of milk glycoproteins have also been shown to exert protection in the developing neonate. Lactoferrin, one of the most abundant glycoproteins in human milk with bacteriostatic and bactericidal functions, is shown to be dynamically fucosylated during the first 10 days of lactation while the total protein level remained unchanged (42). The

glycosylation of lactoferrin has been shown to influence the glycoprotein's susceptibility to proteolysis thus affecting the production of antimicrobial peptides (43). Lactadherin, a milk glycopeptide, has been shown to inhibit the ability of rotavirus to infect MA-104 cells in culture, but not after its sialic acid residue is removed (14). Similar studies testing the protective effects of fucosylated human milk N-glycans have not yet been done. The few human milk N-glycans that have been structurally characterized show α 1,3 and α 1,6 Fuc linkages (44, 45). The α 1,2 Fuc linkages found to be protective as HMO constituents have not yet been found as components of human milk N-glycans. In this study, no structural information was determined for the N-glycans and it is not known whether any fucosylated N-glycans represented here contain α 1,2 Fuc bonds. Structural determination and mechanistic studies are required to determine whether human milk N-glycans exert protection against pathogenesis in the infant.

Sialic Acid Biology

Of human milk N-glycans identified in this study, a large number contained the 9-carbon sialic acid Neu5Ac—both by percent of N-glycan compositions found (38%) and by average abundance percentage (47%). Throughout lactation and between different gestational ages of the infant at birth, human milk was found to contain high levels of Neu5Ac, 69-76% of which is derived from HMO, 21-28% from glycoproteins, 1% from gangliosides and 3% from free Neu5Ac (46). Glycoprotein-bound Neu5Ac in human colostrum is 5 mmol/L and decreases to 1 mmol/L in mature milk (46). A recent comprehensive review of sialic acid research shows that the Neu5Ac in human milk is potentially important for infant brain development and cognition (47). The tissues of the human central nervous system (CNS) are higher in Neu5Ac concentration than any other tissues in the body. Sixty-five percent of the conjugated sialic acids of the CNS reside on cell membrane gangliosides and 32% are attached to glycoproteins (48). Most of the glycoprotein-bound sialic acids are attached to one protein: neural cell adhesion molecules (NCAM) (48), a polysialylated protein widely expressed on CNS cell membranes and shown to be involved in synaptic plasticity, cell-to-cell interactions, neuronal outgrowth and memory formation (47, 49, 50). Supplementation studies in neonatal pigs and rodents with both free and conjugated radio-labeled Neu5Ac show not only uptake of Neu5Ac into brain tissue and incorporation of Neu5Ac into brain glycoproteins and gangliosides but also measurable enhancement of learning and memory (51-53). Though humans possess the requisite enzymes for endogenous Neu5Ac synthesis (54, 55), the rate-limiting enzyme (UDP-N-acetylglucosamine-2-epimerase (UDP-GlcNAc-2-epimerase)/N-acetylmannosamine kinase (ManNAc Kinase)) of this pathway has been shown to exist only at low levels in neonatal rats and guinea pigs (56). Researchers hypothesize that levels of ManNAc Kinase—and thus Neu5Ac synthesis potential—are low in human infants, especially those born prematurely (47, 57). Concurrently, the level of intestinal neuraminidase (which can release Neu5Ac from various glycans) has been shown to be highest in rats and mice during the suckling period (58). Breast milk may provide an important source of Neu5Ac for the neonate in the context of the low capacity for endogenous production and high requirement for the neonatal brain. It is currently unknown which Neu5Ac-containing molecules—HMO, gangliosides or glycoproteins—are the most important exogenous source for brain Neu5Ac. Structural differences between and within these molecular types will result in differences in susceptibility to enzymatic release of the

Neu5Ac (by enzymes such as intestinal sialidases) and, thus, variance in their potential as a Neu5Ac delivery source. More research is required to determine which structures are most conducive to Neu5Ac release. Provision of particular sialylated N-glycans structures may be important for Neu5Ac-enhanced brain development in the neonate, especially in premature infants. Though it is known that total Neu5Ac levels in premature milk are 13-23% higher than in term milk during the first month of lactation (46), it remains to be determined how levels of sialylation compare between term and premature milk N-glycans.

This study was the first to our knowledge to examine in detail the N-glycan composition of glycoproteins in the skim fraction of human milk and distinguish them from free HMO. A novel approach is described for analyzing human milk N-glycans as a distinct set of compounds. Fifty-two N-glycans were discovered in three 3-4 month postpartum milk samples. All N-glycans found had mass errors of less than 6 ppm. Of the 52 N-glycans identified, 65% were fucosylated, 38% were sialylated and 25% were both fucosylated and sialylated. The number of Fuc residues present ranged from 0 to 4 and the number of Neu5Ac residues present ranged from 0 to 2. Of the N-glycans identified, 25 of the N-glycans have not been previously described in association with human milk proteins and 27 have. The three milk samples varied little in terms of specific N-glycan compositions present—the vast majority of N-glycan abundance in each donor sample consisted of N-glycan compositions shared between all three samples. Future areas of study include changes in an individual mother over the course of lactation; comparisons between milks from mothers delivering at term vs. preterm and different metabolic states; and comparisons among other mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Information: This work was supported by: The University of California Discovery Program (05GEB01NHB), the National Institute of Environmental Health Sciences (NIEHS) (P42ES004699), NIEHS R01 ES002710, NIEHS Superfund Research Program P42 ES011269, the California Dairy Research Foundation, the National Science Foundation Graduate Student Research Fellowship, the National Institutes of Health Training Program in Biomolecular Technology 2-T3-GM08799, the Jastro Shields Research Scholarship Award, the Graduate Scholars Fellowship and the CHARGE study (P01 ES11269).

Literature Cited

1. Stanley, P.; Schachter, H.; Taniguchi, N. N-Glycans. In: Varki, A., editor. In Essentials of glycobiology. 2. Vol. 1. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.
2. Shailubhai K, Saxena ES, Balapure A, Vijay I. Developmental regulation of glucosidase I, an enzyme involved in the processing of asparagine-linked glycoproteins in rat mammary gland. J Biol Chem. 1990; 265:9701. [PubMed: 2190984]
3. Chilton BS, Kaplan HA, Lennarz WJ. Estrogen regulation of the central enzymes involved in O- and N-linked glycoprotein assembly in the developing and the adult rabbit endocervix. Endocrinology. 1988; 123:1237. [PubMed: 3136004]
4. Butler M, Quelhas D, Critchley AJ, Carchon H, Hebestreit HF, Hibbert RG, Vilarinho L, Teles E, Matthijs G, Schollen E. Detailed glycan analysis of serum glycoproteins of patients with congenital

disorders of glycosylation indicates the specific defective glycan processing step and provides an insight into pathogenesis. *Glycobiology*. 2003; 13:601. [PubMed: 12773475]

5. Ben-Dor S, Esterman N, Rubin E, Sharon N. Biases and complex patterns in the residues flanking protein N-glycosylation sites. *Glycobiology*. 2004; 14:95. [PubMed: 14514714]
6. Ratner DM, Adams EW, Disney MD, Seeberger PH. Tools for glycomics: mapping interactions of carbohydrates in biological systems. *ChemBioChem*. 2004; 5:1375–1383. [PubMed: 15457538]
7. Zhang XL. Roles of glycans and glycopeptides in immune system and immune-related diseases. *Curr Med Chem*. 2006; 13:1141–1147. [PubMed: 16719775]
8. Dwek, RA. *Chem Rev*. Vol. 96. Washington, DC, U. S.: 1996. *Glycobiology: toward understanding the function of sugars*; p. 683-720.
9. Brines R, Brock J. The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum: unusual resistance of human apolactoferrin to proteolytic digestion. *Biochim Biophys Acta, Gen Subj*. 1983; 759:229–235.
10. Newburg DS. Human milk glycoconjugates that inhibit pathogens. *Curr Med Chem*. 1999; 6:117–128. [PubMed: 9927761]
11. Chaturvedi, P.; Warren, CD.; Buescher, CR.; Pickering, LK.; Newburg, DS.; Center, S. Survival of human milk oligosaccharides in the intestine of infants. In: Newburg, DS., editor. *Bioactive components of human milk*. 1. Vol. 1. Kluwer Academic/Plenum Publishers; New York, NY: 2001. p. 315-323.
12. Ashkenazi S, Newburg D, Cleary T. The effect of human milk on the adherence of enterohemorrhagic *E. coli* to rabbit intestinal cells. *Adv Exp Med Biol*. 1991; 310:173. [PubMed: 1808994]
13. Schrotten H, Hanisch F, Plogmann R, Hacker J, Uhlenbruck G, Nobis-Bosch R, Wahn V. Inhibition of adhesion of S-fimbriated *Escherichia coli* to buccal epithelial cells by human milk fat globule membrane components: a novel aspect of the protective function of mucins in the nonimmunoglobulin fraction. *Infect Immun*. 1992; 60:2893. [PubMed: 1377184]
14. Yolken R, Peterson J, Vonderfecht S, Fouts E, Midthun K, Newburg D. Human milk mucin inhibits rotavirus replication and prevents experimental gastroenteritis. *J Clin Invest*. 1992; 90:1984. [PubMed: 1331178]
15. Landberg E, Huang Y, Strömqvist M, Mechref Y, Hansson L, Lundblad A, Novotny MV, Pålsson P. Changes in Glycosylation of Human Bile-Salt-Stimulated Lipase during Lactation. *Arch Biochem Biophys*. 2000; 377:246–254. [PubMed: 10845701]
16. Charlwood J, Hanrahan S, Tyldesley R, Langridge J, Dwek M, Camilleri P. Use of proteomic methodology for the characterization of human milk fat globular membrane proteins. *Anal Biochem*. 2002; 301:314–324. [PubMed: 11814302]
17. Gustafsson A, Kacs Kovics I, Breimer ME, Hammarström L, Holgersson J. Carbohydrate phenotyping of human and animal milk glycoproteins. *Glycoconjugate J*. 2005; 22:109–118.
18. Davies M, Smith R. Use of a porous graphitised carbon column for the high-performance liquid chromatography of oligosaccharides, alditols and glycopeptides with subsequent mass spectrometry analysis. *J Chromatogr, A*. 1993; 646:317–326.
19. Niñonuevo M, An H, Yin H, Killeen K, Grimm R, Ward R, German B, Lebrilla C. Nanoliquid chromatography mass spectrometry of oligosaccharides employing graphitized carbon chromatography on microchip with a high accuracy mass analyzer. *J Electrophor*. 2005; 26:3641–3649.
20. Kronewitter S, de Leoz M, Peacock K, McBride K, An H, Miyamoto S, Leiserowitz G, Lebrilla C. Human Serum Processing and Analysis Methods for Rapid and Reproducible N-Glycan Mass Profiling. *J Proteome Res*. 2010
21. Chu CS, Niñonuevo MR, Clowers BH, Perkins PD, An HJ, Yin H, Killeen K, Miyamoto S, Grimm R, Lebrilla CB. Profile of native N-linked glycan structures from human serum using high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. *J Proteomics*. 2009; 9:1939–1951.
22. Albrecht S, Schols HA, van den Heuvel EGHM, Voragen AGJ, Gruppen H. CE LIF MSn profiling of oligosaccharides in human milk and feces of breast fed babies. *J Electrophor*. 2010; 31:1264–1273.

23. Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology*. 2001; 11:365. [PubMed: 11425797]
24. Shen Z, Warren CD, Newburg DS. High-Performance Capillary Electrophoresis of Sialylated Oligosaccharides of Human Milk. *Anal Biochem*. 2000; 279:37–45. [PubMed: 10683228]
25. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL, Killeen K, Grimm R. A strategy for annotating the human milk glycome. *J Agric Food Chem*. 2006; 54:7471–7480. [PubMed: 17002410]
26. Kitagawa H, Nakada H, Fukui S, Funakoshi I, Kawasaki T, Yamashina I, Tate S, Inagaki F. Novel oligosaccharides with the sialyl-Lea structure in human milk. *Biochemistry*. 1991; 30:2869–2876. [PubMed: 2007125]
27. Lo Cascio RG, Ninonuevo MR, Freeman SL, Sela DA, Grimm R, Lebrilla CB, Mills DA, German JB. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J Agric Food Chem*. 2007; 55:8914–8919. [PubMed: 17915960]
28. Niñonuevo MR, Perkins PD, Francis J, Lamotte LTM, Lo Cascio RG, Freeman SL, Mills DA, German JB, Grimm R, Lebrilla CB. Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry. *J Agric Food Chem*. 2007; 56:618–626. [PubMed: 18088092]
29. Prieto PA, Smith DF. A new sialyloligosaccharide from human milk: Isolation and characterization using anti-oligosaccharide antibodies. *Arch Biochem Biophys*. 1984; 229:650–656. [PubMed: 6703714]
30. Wu S, Tao N, German JB, Grimm R, Lebrilla CB. The development of an annotated library of neutral human milk oligosaccharides. *J Proteome Res*.
31. Royle L, Campbell MP, Radcliffe CM, White DM, Harvey DJ, Abrahams JL, Kim YG, Henry GW, Shadick NA, Weinblatt ME. HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal Biochem*. 2008; 376:1–12. [PubMed: 18194658]
32. Kim YG, Jang KS, Joo HS, Kim HK, Lee CS, Kim BG. Simultaneous profiling of N-glycans and proteins from human serum using a parallel-column system directly coupled to mass spectrometry. *J Chromatogr, B: Biomed Sci Appl*. 2007; 850:109–119.
33. Davies M, Smith KD, Harbin AM, Hounsell EF. High-performance liquid chromatography of oligosaccharide alditols and glycopeptides on a graphitized carbon column. *J Chromatogr, A*. 1992; 609:125–131.
34. Kronewitter SR, An HJ, de Leoz ML, Lebrilla CB, Miyamoto S, Leiserowitz GS. The development of retrosynthetic glycan libraries to profile and classify the human serum N linked glycome. *J Proteomics*. 2009; 9:2986–2994.
35. Domon B, Costello CE. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J*. 1988; 5:397–409.
36. Marionneau S, Ruvon N, Le Moullac-Vaidye B, Clement M, Cailleau-Thomas A, Ruiz-Palacios G, Huang P, Jiang X, Le Pendu J. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology*. 2002; 122:1967–1977. [PubMed: 12055602]
37. Ruvoen-Clouet N, Ganiere JP, Andre-Fontaine G, Blanchard D, Le Pendu J. Binding of rabbit hemorrhagic disease virus to antigens of the ABH histo-blood group family. *J Virol*. 2000; 74:11950. [PubMed: 11090195]
38. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. *Campylobacter jejuni* binds intestinal H (O) antigen (Fuc 1, 2Gal 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*. 2003; 278:14112. [PubMed: 12562767]
39. Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr*. 2005; 25:37. [PubMed: 16011458]
40. Morrow, AL.; Ruiz-Palacios, GM.; Altaye, M.; Jiang, X.; Lourdes Guerrero, M.; Meinzen-Derr, JK.; Farkas, T.; Chaturvedi, P.; Pickering, LK.; Newburg, DS. *J Pediatr*. Vol. 145. N Y., NY, U S: 2004. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants; p. 297-303.

41. Newburg D, Ruiz-Palacios G, Altaye M, Chaturvedi P, Meinzen-Derr J, Guerrero M, Morrow A. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology*. 2004; 14:253. [PubMed: 14638628]
42. Froehlich J, Dodds E, Barboza M, McJimpsey E, Seipert R, Francis J, An H, Freeman S, German J, Lebrilla C. Glycoprotein Expression in Human Milk during Lactation. *J Agric Food Chem*. 2010; 58:6440–6448. [PubMed: 20415418]
43. van Veen H, Geerts M, van Berkel P, Nuijens J. The role of N-linked glycosylation in the protection of human and bovine lactoferrin against tryptic proteolysis. *Eur J Biochem*. 2004; 271:678–684. [PubMed: 14764083]
44. Vliegenthart JFG, Dorland L, Van Halbeek H. High resolution, ¹H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv Carbohydr Chem Biochem*. 1983; 41:209–374.
45. Mechref Y, Chen P, Novotny M. Structural characterization of the N-linked oligosaccharides in bile salt-stimulated lipase originated from human breast milk. *Glycobiology*. 1999; 9:227. [PubMed: 10024660]
46. Wang B, Brand-Miller J, McVeagh P, Petocz P. Concentration and distribution of sialic acid in human milk and infant formulas. *Am J Clin Nutr*. 2001; 74:510. [PubMed: 11566650]
47. Wang B. Sialic acid is an essential nutrient for brain development and cognition. *Annu Rev Nutr*. 2009; 29:177–222. [PubMed: 19575597]
48. Brunngraber E. Biochemistry, function, and neuropathology of the glycoproteins in brain tissue. *Adv Exp Med Biol*. 1972; 32:109. [PubMed: 4277379]
49. Kiss J, Rougon G. Cell biology of polysialic acid. *Curr Opin Neurobiol*. 1997; 7:640–646. [PubMed: 9384537]
50. Rutishauser U, Landmesser L. Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. *Trends Neurosci*. 1996; 19:422–427. [PubMed: 8888519]
51. Nöhle U, Schauer R. Uptake, metabolism and excretion of orally and intravenously administered, ¹⁴C- and ³H-labeled N-acetylneuraminic acid mixture in the mouse and rat. *Hoppe-Seyler's Z Physiol Chem*. 1981; 362:1495. [PubMed: 7030915]
52. Wang B, Downing J, Petocz P, Brand-Miller J, Bryden W. Metabolic fate of intravenously administered N-acetylneuraminic acid-6-¹⁴C in newborn piglets. *Asia Pac J Clin Nutr*. 2007; 16:110. [PubMed: 17215187]
53. Witt W, von Nicolai H, Zilliken F. Uptake and distribution of orally applied N-acetyl-(¹⁴C) neuraminosyl-lactose and N-acetyl-(¹⁴C) neuraminic acid in the organs of newborn rats. *Nutr Metab*. 1979; 23:51. [PubMed: 759994]
54. Stäsche R, Hinderlich S, Weise C, Effertz K, Lucka L, Moormann P, Reutter W. A bifunctional enzyme catalyzes the first two steps in N-acetylneuraminic acid biosynthesis of rat liver. *J Biol Chem*. 1997; 272:24319. [PubMed: 9305888]
55. Tanner M. The enzymes of sialic acid biosynthesis. *Bioorg Chem*. 2005; 33:216–228. [PubMed: 15888312]
56. Gal B, Ruano M. Developmental changes in UDP-N-acetylglucosamine 2-epimerase activity of rat and guinea-pig liver. *Comp Biochem Physiol, Part B: Biochem Mol Biol*. 1997; 118:13–15.
57. Karim M, Wang B. Is sialic acid in milk food for the brain. *Perspect Agric Vet Sci Nutr Nat Resour*. 2006; 1:18–29.
58. Dickson J, Messer M. Intestinal neuraminidase activity of suckling rats and other mammals. Relationship to the sialic acid content of milk. *Biochem J*. 1978; 170:407. [PubMed: 25066]

Abbreviations Used

PNGase F	Peptidyl N-glycosidase F
Asn	Asparagine
Thr	Threonine

Ser	Serine
N-glycans	N-linked glycans
GlcNAc	N-acetylglucosamine
Man	Mannose
HMO	Human milk oligosaccharides
nanoLC	nanoflow LC
TOF	Time-of-flight
MS	Mass spectrometer
ppm	parts per million
SPE	Solid phase extraction
GCC	Graphitized carbon chromatography
ACN	Acetonitrile
TFA	Trifluoroacetic acid
LC-MS	Liquid Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
FA	Formic acid
m/z	mass/charge
ESI	Electrospray ionization
Q-TOF	Quadrupole Time-of-Flight mass spectrometer
PGC	Porous graphitized carbon
HexNAc	N-acetylhexosamine
Hex	Hexose
Neu5Ac	N-acetylneuraminic acid
Fuc	Fucose
ECC	Extracted compound chromatograms
EIC	Extracted ion chromatograms
NV	Noroviruses
CNS	central nervous system
NCAM	neural cell adhesion molecules
ManNAc Kinase	<i>N</i> -acetylmannosamine kinase

Figure 1.

N-glycan identification strategy. The protein sample is divided into two equal parts and to one is added PNGase F, while the other is left unaltered as a control. The remaining experimental steps are run in parallel on both samples. N-glycans are compounds found at levels greater than or equal to two times higher in the PNGase F-incubated samples compared to the control samples.

Figure 2.
Extracted Compound Chromatogram (ECC) for top N-glycans in the milk of donor A.

Figure 4. Total number of N-glycans in three different individual mothers' milks

Table 1

Top 10 Most Abundant N-glycans Identified, Sorted by Abundance.

Mass	Hex	Fuc	HexNAc	NeuAc	Total abundance	Sample	HMO in lit.	Human milk N-glycan in lit.	
2223.803	5	2	4	4	1	29,998,300	ABC	N	Y
2077.745	5	1	4	4	1	28,393,400	ABC	N	Y
1932.708	5	2	4	4	0	20,782,900	ABC	N	Y
2078.766	5	3	4	4	0	17,406,100	ABC	N	Y
1786.65	5	1	4	4	0	15,293,100	ABC	N	Y
1519.566	3	0	5	0	0	6,983,250	ABC	N	N
1931.688	5	0	4	4	1	6,563,950	ABC	N	Y
1665.624	3	1	5	0	0	5,556,500	ABC	N	N
1827.677	4	1	5	0	0	4,995,410	ABC	N	N
2734.993	6	3	5	5	1	3,566,320	ABC	N	N