

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

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S Supporting Information

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 and be at least 2-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 composed 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

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), and 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, the expression of which 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} 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 peptidyl *N*-glycosidase F (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 data), and secretory immunoglobulin A (M. Barboza et al., unpublished data) 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

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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 liquid chromatography (nanoLC) with a graphitized carbon chip and orthogonal time-of-flight (TOF) mass spectrometry for its ability to separate and detect heterogeneous mixtures with high-resolution peak separation and high mass accuracy (routinely between 1 and 6 ppm mass error for complex carbohydrates).^{18,19} High repeatability in retention times, mass assignments, and high mass accuracy make this technique excellent for *N*-glycan profiling of human milk.

MATERIALS AND METHODS

Human Milk Samples. Human milk samples were collected from three healthy, volunteer mothers who had given birth at term. Mature milk from donor A was collected at 3 months postpartum, whereas 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 2200g for 15 min. The supernatant cream layer was removed with a metal spatula. Remaining skim milk infranates were centrifuged for 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 0.5 mL of doubly skimmed milk sample, vortexed for 5 s, centrifuged for 5 s, and then stored at -80°C for 1 h to allow protein precipitation. Samples were then centrifuged at 16000g at 4°C for 15 min. The ethanol–water phase supernatant was removed by pipet, leaving the precipitated proteins.

Release of N-Linked Glycans from Human Milk by *N*-Glycosidase F Digestion. Two milligrams of precipitated protein

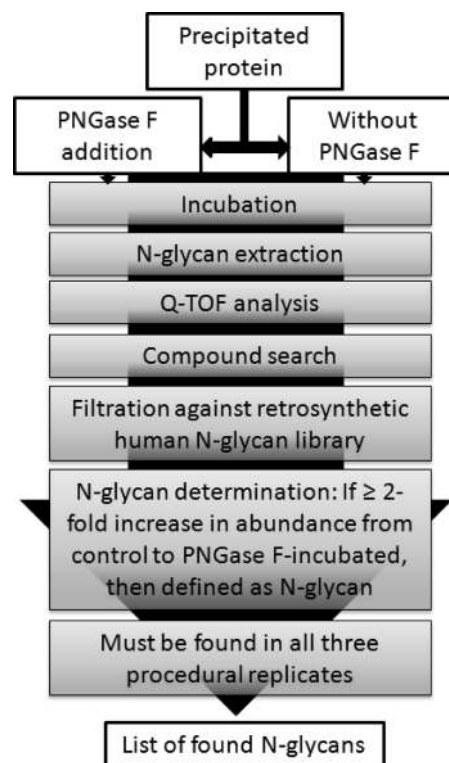


Figure 1. *N*-Glycan identification strategy. The protein sample is divided into two equal parts, and to one is added PNGase F, whereas the other is left unaltered as a control. The remaining experimental steps are run in parallel on both samples. *N*-Glycans are compounds found at levels ≥ 2 times higher in the PNGase F-incubated samples compared to the control samples.

from each sample was added to a 1.5 mL tube (Sarstedt). To this were added 200 μL of nanopure water (18.2 Ω , E-Pure, Barnstead), 200 μL of 200 mM ammonium bicarbonate (Fisher Scientific), and 10 mM dithiothreitol (Sigma Aldrich). This mixture was vortexed and pipet 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 requiring 15–20 μL). Each sample was then divided into two equal parts (Figure 1). Two microliters of *N*-glycosidase F (PNGase F) (New England Biolabs, glycerol free) was added to one of each sample set, creating an experimental and control sample pair. All samples were then placed in a microwave reactor (CEM Corp., Matthews, NC) for 10 min at 20 W 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 μL 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 of nanopure water with 20 s 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 of nanopure water, then conditioned with 6 mL of 80% acetonitrile

(ACN) (Fisher Scientific, HPLC grade) and 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 of freestanding sterile, silicon-sealed cryovial tubes (C-405, Phenix Research Products). Eluted fractions were immediately dried by centrifuge evaporation at 40 °C maximum temperature (typically requiring 3 h) 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 s to resolubilize. Three microliters for each of the 10, 20, and 40% ACN fractions from each sample set was 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 method²¹ 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 an 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; from 16 to 44% B, 20–30 min; from 44 to 100% B, 30–35 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 of nitrogen and 2.0 L of 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 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 <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 laboratory processing was replicated three 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 six 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, because higher charges occur rarely in this context. Peaks of <0.01% relative height or <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 ppm (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 because 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 three 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*-glycans rather than HMOs.

First, compounds matching the retrosynthetic *N*-glycan list were further filtered by a 2-fold abundance difference cutoff (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 of >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 <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

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 <i>N</i> -glycan in lit. |
|----------|-----|-----|--------|-------|-----------------|--------|-------------|-------------------------------------|
| 2223.803 | 5 | 2 | 4 | 1 | 29,998,300 | ABC | N | Y |
| 2077.745 | 5 | 1 | 4 | 1 | 28,393,400 | ABC | N | Y |
| 1932.708 | 5 | 2 | 4 | 0 | 20,782,900 | ABC | N | Y |
| 2078.766 | 5 | 3 | 4 | 0 | 17,406,100 | ABC | N | Y |
| 1786.65 | 5 | 1 | 4 | 0 | 15,293,100 | ABC | N | Y |
| 1519.566 | 3 | 0 | 5 | 0 | 6,983,250 | ABC | N | N |
| 1931.688 | 5 | 0 | 4 | 1 | 6,563,950 | ABC | N | Y |
| 1665.624 | 3 | 1 | 5 | 0 | 5,556,500 | ABC | N | N |
| 1827.677 | 4 | 1 | 5 | 0 | 4,995,410 | ABC | N | N |
| 2734.993 | 6 | 3 | 5 | 1 | 3,566,320 | ABC | N | N |

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. 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 passing the test for all procedural replicates, the abundance is calculated as the average of abundances among 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 by 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 2-fold higher in the PNGase F samples compared with the control samples and, thus, were defined as *N*-glycans in this study (Supporting Information Table S1). Seventeen (77%) were found at abundance levels <2-fold higher in the PNGase F samples compared to the control samples and, thus, were defined as free HMO (data not shown).

Verification of Nonambiguous *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 the 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 three 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 the 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 2 *N*-acetylhexosamine (HexNAc) added to the 3 hexose (Hex) 2 HexNAc *N*-glycan core) to 18 residues and in mass from 1234.43 Da (5-Hex 2-HexNAc) to 3246.18 Da (7-Hex 4-Fucose (Fuc) 6-HexNAc 1-*N*-acetylneuraminic acid (Neu5Ac)). The ranges of monosaccharides in the *N*-glycans identified ranged from 3 to 9 for Hex, from 2 to 6 for HexNAc, from 0 to 4 for Fuc, and from 0 to 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.

Abundances 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, 7 (21%) had three, and 3 (9%) had four fucosyl residues. By abundance, 36% of the fucosylated *N*-glycans had one Fuc, 32% had two, 14% had three, and 2% had

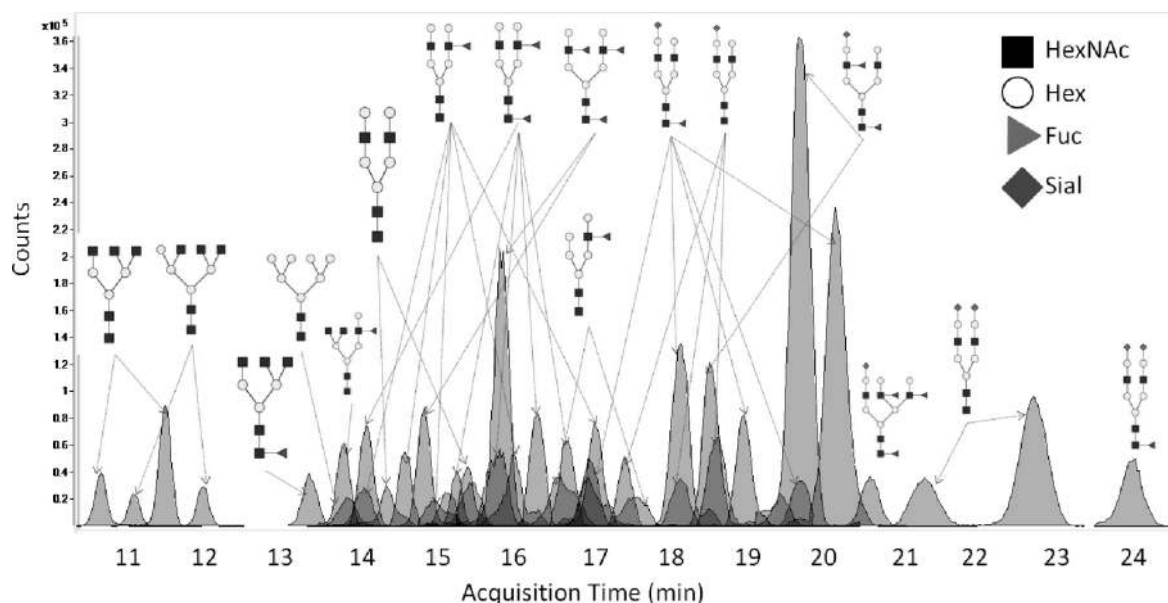


Figure 2. Extracted compound chromatogram for top *N*-glycans in the milk of donor A.

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 monosialylated and 3% was made up of disialylated *N*-glycans.

Individual *N*-Glycan Chromatograms. A software package (MassHunter Qualitative Analysis, Agilent) performed advanced peak finding for the 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 the 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 in 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 (m/z 894.33) are shown in Figure 3A to illustrate the number of isomers that may be found for a single composition. For this composition, six peaks eluting at different retention times were found, suggesting that at least six 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 α or β anomers²¹). 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 library³⁴ 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, D).³⁵ Each of the three proposed structures here demonstrates 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 intermother 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 14 *N*-glycans

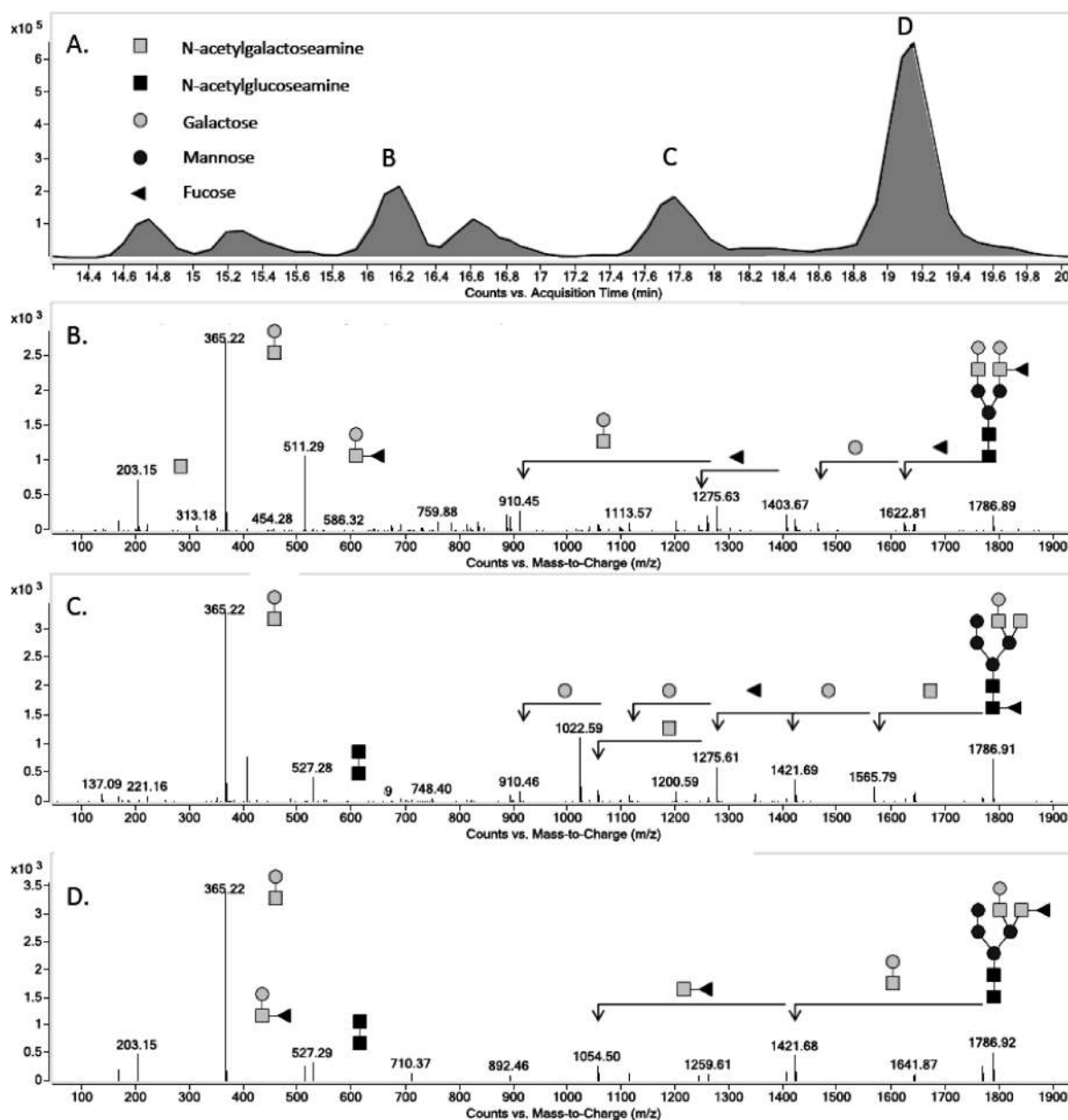


Figure 3. (A) ECCs for 5 Hex 4 HexNAc and 1 dHex (m/z 894.33) in the *N*-glycan extracts of donor milk A. (B–D) Fragmentation spectra for three isomers at 14.706 min (B), 15.163 min (C), and 16.059 min (D).

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 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

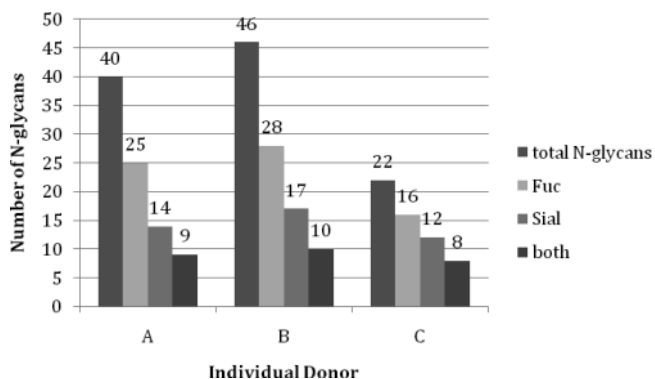


Figure 4. Total number of *N*-glycans in three different individual mothers' milks.

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 radiolabeled 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} Although 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. Although 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 <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 among 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 versus preterm and different metabolic states, and comparisons among other mammals.

■ ASSOCIATED CONTENT

S Supporting Information. Table of 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>.

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Notes

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

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■ 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, N-acetylmannosamine kinase.

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