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Lacto-*N*-Tetraose, Fucosylation, and Secretor Status Are Highly Variable in Human Milk Oligosaccharides From Women Delivering Preterm

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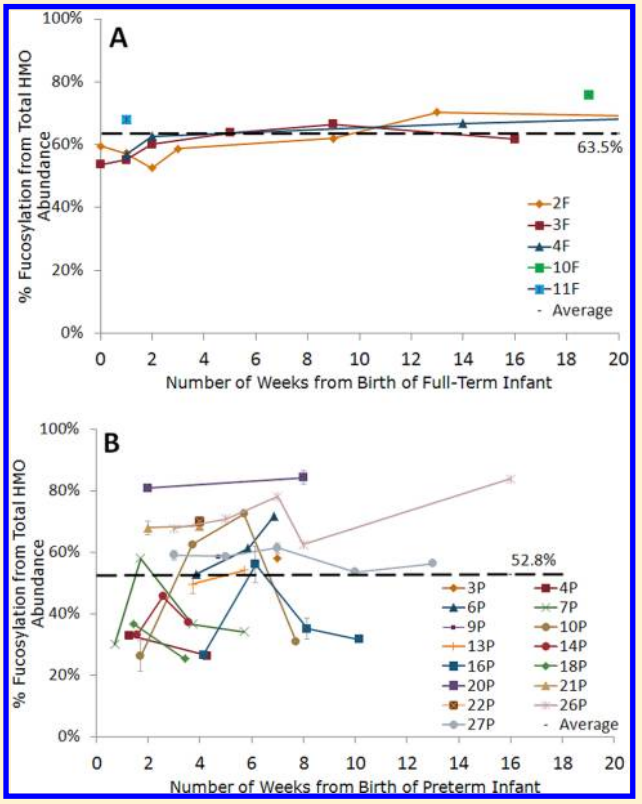
ABSTRACT: Breast milk is the ideal nutrition for term infants but must be supplemented to provide adequate growth for most premature infants. Human milk oligosaccharides (HMOs) are remarkably abundant and diverse in breast milk and yet provide no nutritive value to the infant. HMOs appear to have at least two major functions: prebiotic activity (stimulation of the growth of commensal bacteria in the gut) and protection against pathogens. Investigations of HMOs in milk from women delivering preterm have been limited. We present the first detailed mass spectrometric analysis of the fucosylation and sialylation in HMOs in serial specimens of milk from 15 women delivering preterm and 7 women delivering at term using nanohigh performance liquid chromatography chip/time-of-flight mass spectrometry. A mixed-effects model with Levene's test was used for the statistical analyses. We find that lacto-*N*-tetraose, a core HMO, is both more abundant and more highly variable in the milk of women delivering preterm. Furthermore, fucosylation in preterm milk is not as well regulated as in term milk, resulting in higher within and between mother variation in women delivering preterm vs term. Of particular clinical interest, the α1,2-linked fucosylated oligosaccharide 2′-fucosyllactose, an indicator of secretor status, is not consistently present across lactation of several mothers that delivered preterm. The immaturity of HMO production does not appear to resolve over the time of lactation and may have relevance to the susceptibility of premature infants to necrotizing enterocolitis, late onset sepsis, and related neurodevelopmental impairments.



KEYWORDS: *HMO, LNT, mass spectrometry, sialic acid, sialylation, fucosyllactose, premature infant*

INTRODUCTION

Breast milk is the ideal nutrition for term infants for growth, development, and protection from infections. Human milk from women delivering preterm is not nutritionally adequate for small premature infants and must be fortified to ensure adequate growth in this evolutionarily "new" population.[1] Preterm milk is initially higher in protein, carbohydrate, and sodium than term milk, but the protein content decreases over time and is not adequate for the rapid growth requirements of infants born before 32 weeks gestation.[2] Current human milk fortifiers provide additional protein, fat, vitamins, and a variety of electrolytes and minerals. The fortification of preterm milk with concentrated pooled donor human milk is an intriguing option with potential benefits for growth and protection from infections.[3] The wide variation of preterm milk composition between women and in a given woman over time has prompted proposals to individualize human milk fortification for this population.[4]

Human milk oligosaccharides (HMOs) are unbound sugars with diverse and complicated structures. HMOs commonly have a lactose core (Galβ-1,4Glc) at the reducing end that can be elongated with up to 25 *N*-acetyllactosamine repeat units (Galβ1−3/4GlcNAc). The oligosaccharide backbone can be

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sialylated in α2−3 or α2−6 linkages and/or fucosylated in α1−2, α1−3, or α1−4 linkages.[5,6] Humans produce greater numbers and more complex structures of oligosaccharides than any other mammal with many women producing more than 100 different structures.[7] A survey of nine small HMOs in over 500 milk samples from 435 donors in 10 countries demonstrated regional differences in several HMOs and striking similarities in others.[8]

Because the human gut lacks the enzymes to deconstruct these compounds, they do not provide any nutritive value for the infant and yet they are the third most abundant solid component in human milk.[5] This raises the question of HMO function given that significant energy is required for the mammary gland to synthesize these molecules. Evidence suggests at least two major functions.

First, HMOs promote growth of specific strains of beneficial bacteria such as bifidobacteria.[9,10] The ability of bacteria to deconstruct HMOs is encoded in the bacterial genome and varies among bifidobacterial strains, suggesting the coevolution of human lactation and specific commensal organisms.[10] In other words, the primary function of HMOs appears to be to nourish specific strains of bacteria allowing them to flourish. In this way, the mother's milk shapes the intestinal microbiota of her infant.

Second, HMOs (especially fucosylated species) act as free-floating receptor analogs, competing for bacterial binding in the intestinal lumen to prevent intestinal pathogen adhesion to epithelial surfaces.[11] HMOs and cell surface glycoforms are synthesized by similar glycosyltransferases and thus have common epitopes. Inhibition of binding to cell surfaces by HMOs has been demonstrated for organisms that commonly cause sepsis (e.g., *Streptococcus pneumonia*[12] and *Listeria monocytogenes*[13]) and diarrhea (e.g., enteropathogenic *E. coli*,[14] *Campylobacter jejuni*,[15] and Norovirus[16]) in infants. HMOs have been shown in animal models to decrease toxin-induced diarrhea.[17,18]

In addition to these major functions, a role for sialylated oligosaccharides, that is, *N*-acetylneuraminic acid-containing oligosaccharides, in early brain growth has been postulated from animal studies.[19,20] Sialic acids increase the production of gangliosides, which are important components of membrane receptors and cell surfaces of the nervous system.[21] The precise role of dietary sialic acid, if any, in human brain development is unclear. Studies of ^{13}C-labeled galactose ingested by mothers during lactation suggest that some HMOs are absorbed intact from the intestinal tract with limited data suggesting a role for HMOs in leukocyte adhesion and platelet−neutrophil interactions.[22]

Proportions of fucosylated and sialylated HMOs in human term milk are generally 60−80% and 10−15%, respectively,[5] and do not vary significantly at different stages of lactation.[23] Analysis of HMOs in preterm milk has been limited. Early studies suggested colostral preterm milk contains a higher amount of oligosaccharides than term milk with the amount of oligosaccharides decreasing during the course of lactation in preterm milk.[24,25] Studies of limited numbers of HMOs have shown wide variation between individuals with no significant differences in 10 neutral oligosaccharides between preterm and term milk,[26] higher sialylated HMO content in preterm milk than term milk that persists over time,[27] and significant variation among premature infants in 23 HMOs based on secretor status and Lewis antigen status.[28] In milk from mothers delivering at term, monitoring of hundreds of structures indicated that the amount of oligosaccharides and particularly those important for beneficial bacteria remain relatively constant over the lactation period.[23]

In this study, we use mass spectrometry to profile the temporal and individual variations of overall abundance as well as fucosylation and sialylation of HMOs in preterm and term milk. Specifically, we use matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI FTICR MS) to profile the preterm and term milk samples and nanohigh performance liquid chip/time-of-flight (nano-HPLC Chip/TOF) MS to investigate the temporal and individual variations of fucosylation and sialylation in preterm and term milk. While both instruments provide high sensitivity and high mass accuracy (<5 ppm), the latter provides an added dimension of reproducible retention times for each oligosaccharide, allowing the monitoring of hundreds of structures.[5,23] HMOs were characterized using an in-house library for neutral[6] and sialylated[29] HMOs based on mass-to-charge ratios (m/z) and retention time values.

MATERIALS AND METHODS

Milk Sample Collection and Processing

Milk samples from 15 mothers delivering prematurely were collected in the Neonatal Intensive Care Unit at UC Davis Children's Hospital. Milk samples were also collected from 7 mothers of healthy term infants following discharge from the hospital. The UC Davis Institutional Review Board approved all aspects of the study and informed consent was obtained from all subjects. The samples were collected in standard collection tubes and frozen at −80 °C prior to extraction. Samples were labeled with randomly generated numbers at the time of collection to protect patient privacy and ensure blinding during analysis.

Extraction and Purification of Human Milk Oligosaccharides (HMOs)

HMOs were extracted and purified as described previously.[5,23] Briefly, 500 μL of thawed milk was centrifuged for 30 min at 4 °C. The top fat layer was removed and to the decanted skimmed milk were added four volumes of chloroform/methanol (2:1 by volume). The mixture was then centrifuged at 4000× *g* for 30 min at 4 °C and the upper layer was carefully transferred to another vial. Two volumes of ethanol were added to the upper layer and the mixture was left at 4 °C overnight, then centrifuged for 30 min at 4 °C. The supernatant solution was evaporated to dryness using a centrifugal evaporator (Savant AES 2010).

To prevent double peaks due to the beta and alpha anomers of a single HMO, the HMOs were reduced to alditol form by adding 1.0 M sodium borohydride and incubating at 65 °C for 1 h (or 42 °C overnight). HMOs were desalted and purified by graphitized carbon solid-phase extraction using 20% acetonitrile (ACN) and 40% ACN with 0.05% trifluoroacetic acid (TFA). Cartridges were cleaned and conditioned using nanopure water, 80% ACN, and nanopure water. Samples were then loaded to the cartridges, desalted with water, and eluted with 20 and 40% ACN. Eluted ACN fractions were evaporated to dryness. Samples were reconstituted with nanopure water prior to mass spectrometry analysis.

MALDI-FTICR MS Analysis

Mass spectra were recorded on an FTICR MS with an external source ProMALDI (Varian, Palo Alto, CA) equipped with a 7.0

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Table 1. Percentage of LNT, Fucosylation, and Sialylation in the Free HMOs in Milk Samples Collected from 15 Mothers who Delivered Preterm[a]

ID	BW (g)	GA (weeks)	PMA (weeks)	% Sia	% Fuc	% mono Fuc	% di Fuc	% tri Fuc	% tetra Fuc	% 2′FL	2′ FL mass	2′ FL mass error, ppm	% 3-FL	% LNT
3P	570	25	32	26.1	58.0	71.4	25.2	3.4	0.0	0.1	490.1898	0.204	0.14	21.2
4P	1120	27	28	23.7	32.9	80.2	16.5	3.1	0.1	0.0	490.1899	0.408	0.03	43.1
			31	27.9	26.2	83.4	14.8	1.8	0.0	0.1	490.1897	0.000	0.03	48.8
6P	1170	27	31	8.4	52.8	84.0	14.8	1.2	0.0	5.6	490.1915	3.672	ND	35.0
			33	7.6	61.2	75.9	21.3	2.5	0.3	7.4	490.1912	3.060	ND	26.0
			34	6.2	71.6	79.8	16.6	3.3	0.3	19.1	490.1901	0.816	0.06	19.3
7P	1450	30	31	3.6	30.0	87.3	12.5	0.3	0.0	ND	ND	ND	0.01	52.5
			32	9.9	58.0	76.6	21.0	2.3	0.0	0.3	490.1891	1.224	ND	33.9
			34	8.3	36.6	67.9	30.1	2.0	0.0	0.3	490.1883	2.856	0.06	51.5
			36	17.3	34.1	75.6	20.3	4.1	0.0	0.7	490.1900	0.612	ND	35.9
9P	1030	26	31	21.7	58.6	63.6	34.4	1.9	0.1	16.1	490.1903	1.224	0.04	13.1
10P	920	28	30	16.3	26.1	67.1	30.0	2.4	0.1	0.6	490.1907	2.040	ND	28.9
			32	6.2	62.5	87.6	10.9	1.5	0.1	16.3	490.1895	0.408	ND	26.9
			34	3.9	72.5	86.3	12.5	1.1	0.1	28.4	490.1893	0.816	ND	20.6
			36	6.5	30.7	60.0	37.7	2.2	0.1	3.9	490.1895	0.408	ND	46.3
13P	570	24	28	17.9	49.5	55.0	41.2	3.5	0.3	7.9	490.1906	1.836	ND	23.6
			30	10.3	54.0	58.3	35.8	4.5	0.7	5.4	490.1903	1.224	ND	20.1
14P	1105	28	30	22.0	33.1	80.8	16.8	2.3	0.1	0.1	490.1902	1.020	ND	29.1
			31	13.8	45.6	84.0	15.4	0.6	0.0	2.6	490.1883	2.856	ND	28.7
			32	22.7	37.1	72.5	21.9	4.4	0.2	0.3	490.1908	2.244	ND	29.7
16P	660	25	29	12.7	26.6	90.1	9.2	0.7	0.0	0.1	490.1908	2.244	ND	48.3
			31	6.4	56.0	80.5	18.2	1.4	0.0	0.2	490.1892	1.020	ND	32.5
			33	15.6	35.1	50.4	41.8	7.6	0.1	0.1	490.1900	0.612	ND	39.8
			35	13.3	31.6	62.9	31.7	5.4	0.0	0.2	490.1902	1.020	ND	42.3
18P	930	25	26	25.1	36.7	81.4	14.6	3.8	0.2	0.2	490.1904	1.428	ND	31.1
			28	21.2	25.3	84.0	14.5	1.6	0.0	0.2	490.1899	0.408	ND	37.6
20P	900	29	31	6.1	80.8	81.4	16.3	2.0	0.3	40.4	490.1894	0.612	ND	11.7
			37	5.0	84.2	78.9	19.2	1.7	0.2	40.7	490.1898	0.204	ND	10.1
21P	1060	29	31	10.5	67.9	86.0	11.8	2.0	0.1	29.8	490.1898	0.204	0.09	14.9
			33	12.8	68.5	82.1	14.8	3.0	0.2	27.1	490.1882	3.060	0.04	14.7
22P	490	23	27	11.7	70.0	71.4	26.1	2.2	0.3	34.3	490.1879	3.672	ND	13.3
26P	1070	28	31	11.4	67.6	77.0	18.4	4.2	0.4	25.8	490.1899	0.408	0.05	16.7
			33	9.8	70.8	76.1	18.3	5.0	0.5	23.5	490.1897	0.000	0.02	14.3
			35	8.9	78.0	63.4	29.9	5.6	1.1	28.5	490.1908	2.244	0.11	12.8
			36	9.1	62.4	69.8	27.0	3.2	0.0	0.2	490.1897	0.000	0.51	23.4
			44	8.8	83.8	62.5	31.1	5.3	1.1	34.0	490.1907	2.040	0.36	7.7
27P	940	28	31	11.8	58.9	73.3	25.3	1.4	0.0	0.1	490.1889	1.632	0.16	23.2
			33	11.1	58.6	73.8	24.1	2.1	0.0	0.4	490.1891	1.326	0.37	20.8
			35	8.5	61.3	69.9	23.9	2.1	0.3	10.4	490.1913	3.264	0.09	12.3
			38	12.4	53.6	63.8	32.3	3.9	0.1	0.0	490.1898	0.102	0.44	30.7
			41	7.7	56.3	59.9	34.7	5.2	0.2	0.1	490.1893	0.816	0.71	30.2

[a]ID = premature mother number, GA = gestational age at birth, PMA = postmenstrual age (the gestational age at birth plus the chronological age in weeks at the time of milk collection). %Fuc and %Sia are the percent fucosylation and sialylation, respectively, normalized to the total HMO abundance per sample. Percent monofucosylation, difucosylation, trifucosylation and tetrafucosylation are in columns marked as %Mono Fuc, %Di Fuc, %Tri Fuc, %Tetra Fuc correspondingly and refer to the percentages of HMOs with one, two, three and four fucoses, respectively. The percent abundance of 2′-fucosyllactose, an oligosaccharide secretor status marker bearing the alpha-1,2-fucose, is shown in the column %2′-FL, along with its observed neutral mass from nano-LC Chip/TOF MS and mass error in parts per million (ppm). %3-FL = percentage of 3-fucosyllactose. %LNT = percentage of lacto-N-tetraose. ND = not detected.

T magnet, as described previously.[30,31] External calibration was performed using maltooligosaccharides,[32] allowing mass accuracy of 10 ppm or better. 2,5-dihydroxy-benzoic acid was used as a matrix (5 mg/100 μL in 50% ACN:H_2O) for both positive and negative modes. Sodium chloride (0.01 M in 50% ACN:H_2O) was used as a cation dopant for the positive ion mode. The HMOs in the 20% fraction were detected as $[M+Na]^+$ ions in the positive ionization mode while the HMOs in the 40% aqueous ACN fraction were analyzed in the negative ionization mode as $[M-H]^-$ ions.

Milk samples were also analyzed by nano-LC Chip/TOF MS, which adds another dimension of separation based on how the oligosaccharides are retained in the porous graphitized carbon column. For example, a single HMO peak at m/z 1243 $[M+Na]^+$ (neutral mass of 1220) on MALDI FTICR MS analysis separates into five isomeric peaks with distinct retention times on nano-LC Chip/TOF MS analysis: MFLNH I (monofucosyllacto-*N*-hexaose) at 17.70 min, MFLNH III at 17.29 min, MFpLNH IV (monofucosyl-paralacto-N-hexaose) at 15.64 min, IFLNH I (fucosyl-

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Table 2. Percentage LNT, Fucosylation and Sialylation in the HMOs in Milk Samples from Seven Mothers Who Delivered at Term[a]

ID	BW (g)	GA (weeks)	age (weeks)	% Sia	% Fuc	% mono Fuc	% di Fuc	% tri Fuc	% tetra Fuc	% 2′FL	2′FL Mass	2′FL mass error, ppm	% 3′-FL	% LNT
2F	3288	40	0	8.5	59.6	79.0	17.6	2.8	0.6	21.4	490.1881	3.264	0.10	19.2
			1	20.1	57.3	74.3	20.4	4.5	0.8	14.0	490.1881	3.264	0.09	13.5
			2	15.5	52.5	79.6	18.2	1.7	0.5	10.9	490.1864	6.732	ND	14.8
			3	15.9	58.8	76.8	18.9	3.6	0.6	16.0	490.1884	2.652	0.16	13.6
			9	10.3	62.1	80.8	18.0	1.2	0.0	23.6	490.1876	4.284	0.11	12.4
			13	7.8	70.3	73.2	22.2	3.8	0.8	24.7	490.1886	2.244	0.75	15.5
			35	10.8	67.1	75.1	21.9	2.7	0.4	23.3	490.1878	3.876	1.52	16.1
3F	>2500	39	0	19.3	53.6	79.0	17.6	2.8	0.6	21.4	490.1881	3.264	0.03	19.1
			1	24.8	55.2	69.6	22.7	6.4	1.3	16.9	490.1878	3.876	0.04	16.8
			2	20.8	60.2	74.7	19.4	4.9	0.9	16.7	490.1879	3.672	0.08	15.1
			5	18.4	63.8	73.1	20.1	5.5	1.3	19.5	490.1877	4.080	0.12	14.7
			9	21.5	66.5	69.4	23.9	5.1	1.6	21.3	490.1882	3.060	0.25	14.0
			16	19.3	61.7	76.5	18.3	4.5	0.8	20.6	490.1881	3.264	0.21	15.1
4F	3815	40	1	20.8	56.8	77.8	17.2	4.3	0.6	14.1	490.1875	4.488	0.04	15.8
			2	18.3	62.7	79.0	17.6	2.8	0.6	17.0	490.1878	3.876	0.07	15.4
			14	11.7	66.7	77.1	19.1	3.2	0.6	25.1	490.1884	2.652	0.33	15.7
			21	13.4	68.3	74.1	21.4	3.8	0.7	29.1	490.1880	3.468	0.51	15.8
			30	10.2	70.0	74.6	21.9	3.0	0.5	33.3	490.1881	3.264	0.90	16.3
			39	11.7	70.0	71.4	26.1	2.2	0.3	34.3	490.1879	3.672	0.82	15.3
10F	3318	40	19	4.9	75.8	79.2	17.1	3.3	0.3	39.4	490.1898	−0.204	0.25	14.6
11F	4000	38	10	9.0	68.0	61.7	30.9	6.1	1.2	10.2	490.1900	−0.612	0.55	24.8
12F	4000	40	>0	6.2	68.9	62.9	33.2	3.4	0.5	19.1	490.1904	−1.428	0.93	21.5
13F	3227	40	>0	10.4	65.1	70.7	26.0	3.3	0.0	0.1	490.1898	−0.204	1.16	24.0

[a]Column labels are the same as in Table 1, except that age is the age of the infant at milk collection.

paralacto-N-hexaose) at 21.55 min, and IFLNH III at 18.44 min.[6]

Nano-HPLC Chip/TOF MS Analysis

HMO fractions were pooled and analyzed using an Agilent 6200 Series HPLC Chip/TOF-MS system as described previously.[23] Briefly, separation was performed using a binary gradient solvent system consisting of A: 3% ACN in 0.1% formic acid solution, and B: 90% ACN in 0.1% formic acid solution. The column was initially equilibrated and eluted at a flow rate of 0.4 μL for nanopump and 4 μL for capillary pump. The gradient ran for 65 min and was programmed as follows: 2.5−20 min: 0−16% B; 20−30 min: 16−44%B; 30−35 min: B increased to 100%; 35−45 min: continue at 100% B; and 45−65 min: 0% B to equilibrate the chip column before next sample injection.

Data Preparation and Statistical Analysis

Identification of HMOs was performed based on accurate masses and retention times using the HMO library developed by Wu et al.[6,29] Fucosylation and sialylation percentage values were calculated by adding abundances of all fucosylated and sialylated oligosaccharide species, respectively, and normalizing the values with the total HMO abundance per sample. Mono-, di-, tri- and tetrafucosylation was determined by adding the abundances of oligosaccharides containing 1, 2, 3, and 4 fucose residues, respectively, and normalizing the values either with the total HMO abundance or total HMO fucosylation abundance. Percentages of 2′-fucosyllactose and 3-fucosyllactose were determined by normalizing against the total HMO abundance. Percentages of 3-fucosyllactose below the detection limit of 0.0010% were replaced with a value of 0.00005%. Reported results were substantively the same as results from alternative imputation procedures, including replacing non-detectable values with (i) the minimum (0.000%) or (ii) maximum (0.0010%) possible nondetectable value or with (iii) randomly imputed values within the nondetectable range.

Due to the small sample size and variable number of samples from different mothers, we used heterogeneous variance mixed-effects models to model the mean levels and variances in HMO abundance values over time in mothers delivering at term and preterm.[33] Mean levels were modeled with fixed effects for gestation group (full-term vs preterm) and postmenstrual age (PMA) at collection (specified as a linear term to assess overtime changes and centered at 40 weeks to aid in interpretation) and with interactions of the fixed effects included if statistical significance testing indicated that overtime changes in mean levels varied by group. The variation of the marginal residuals (deviations of the actual observations from the fixed effects mean model) was modeled as the sum of mother-specific random effects (to account for between-mother differences in mean marginal residual levels) and conditional residual errors (to account for within-mother, overtime variations). Models were specified with gestation group-specific variance components and estimated by restricted maximum likelihood estimation using Version 9.2 of the SAS System.[34] Levene's testing procedure was used to compare full and preterm mothers on between-mother and within-mother, overtime variance components, with the procedure applied to absolute values of empirical best linear unbiased predictions of the random effects and to absolute values of estimated conditional residuals, respectively.[33] To aid interpretation of the amount of between-mother and within-mother variation, the group-specific mean absolute deviations (MAD) compared by Levene's test are reported for select outcomes. Group-specific adjusted standard deviations represent the square root

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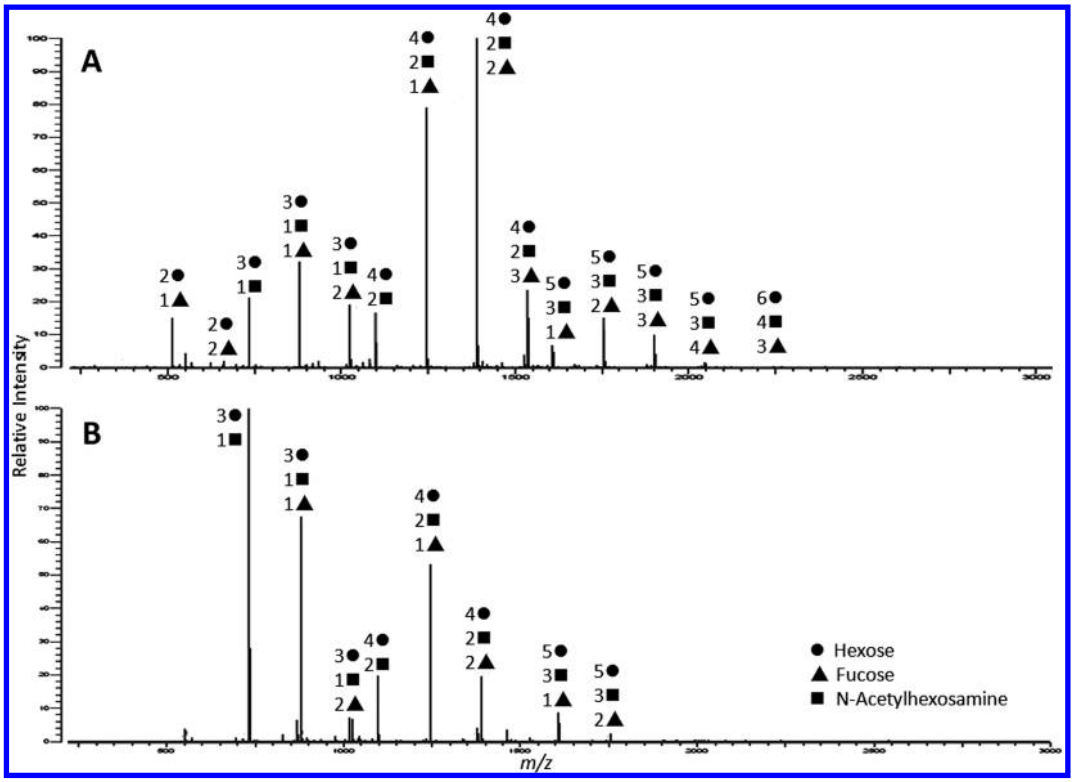

Figure 1. MALDI FT-ICR (+) MS profiles of neutral human milk oligosaccharides isolated from human milk of mothers who delivered (A) at term and (B) preterm. Samples were analyzed in the positive ionization mode.

of the sum of the variance components estimated in the mixed-effects model.

RESULTS

Preterm Milk and Term Milk HMO Profiling

Table 1 summarizes preterm milk samples obtained from 15 mothers who delivered infants from 23 to 30 weeks of gestation. Some mothers provided several samples across lactation, giving a total of 41 milk samples. Seven mothers delivered preterm infants with birth weight <1000 g and eight mothers delivered infants with birth weight 1000−1500 g. Table 2 presents similar data for 23 human milk samples obtained from seven mothers who delivered full-term (after 37 weeks).

MALDI FTICR MS analysis suggested that many of the preterm milk samples had less diversity (fewer HMO peaks) than the milk from term mothers. Figure 1 demonstrates two typical spectra for HMOs isolated from term (Figure 1A) and preterm (Figure 1B) milk. Nano-LC Chip/TOF MS analysis demonstrated possible HMO peaks for 41 milk samples from women delivering preterm (mean 146 peaks; range 79−293) and 23 term milk samples (mean 200 peaks range 100−279). Adjusted mean levels of total number of HMOs at gestational age 40 were similar in both groups. One HMO, lacto-N-tetraose (LNT), was significantly less abundant in term milk than preterm milk (adjusted full-term vs preterm mean difference −7.25%, 95% CI: −14.45% to 0.04%). LNT is a core HMO structure and is metabolized by strains of bifidobacteria that are common in infants but not by strains found in adults.[35] A higher degree of variability for LNT was seen in the samples from mothers delivering preterm than at term. The mean absolute deviation (MAD) is a robust measure of this variability. The LNT MADs for between-mother (7.8% vs 3.4%; $p = 0.005$) and within-mother errors (4.6% vs 1.1%; $p < 0.001$) were statistically significantly higher in the preterm group compared to the full term group.

There were also differences between the two groups in the changes in number of HMOs over time. The milk samples from mothers delivering preterm increased by an average of 4 HMOs per week (preterm slope = 4.2, 95% confidence intervals (CI): 0.1 to 8.3), while the milk samples from mothers delivering at term decreased by an average of 2 HMOs per week (full-term slope = −2.1, 95% CI: −3.9 to −0.3). This pattern may suggest that the time of term delivery is the acme of new HMO production, similar to fetal and placental processes influencing rate of growth (growth of the fetus slows and even regresses if delivery is delayed significantly beyond the due date). The increase in the preterm group may have clinical relevance; for example, an infant born at 26 weeks and receiving her mother's milk would potentially receive about 56 new HMOs by 40 weeks PMA that were not present at birth. Variation in number of HMOs within the two groups (pre vs full term) was not significantly different either between mothers or within mothers over time.

Total Fucosylation and Sialylation in Preterm and Term Milk

Figures 2 and 3 present the percentage of fucosylated HMOs for individual mothers delivering preterm and at term. There was no difference in percentage of fucosylated HMOs between term and preterm milk samples (adjusted term vs preterm mean difference at 40 weeks PMA = 6.6%, 95% CI: −3.2% to 16%). For percentage fucosylation, the between-mothers MAD was 10% for the preterm mothers and 2.7% for the term mothers ($p < 0.001$), and the within-mothers MAD (individual variation over time) was 7.3% and 2.7% respectively ($p < 0.001$).

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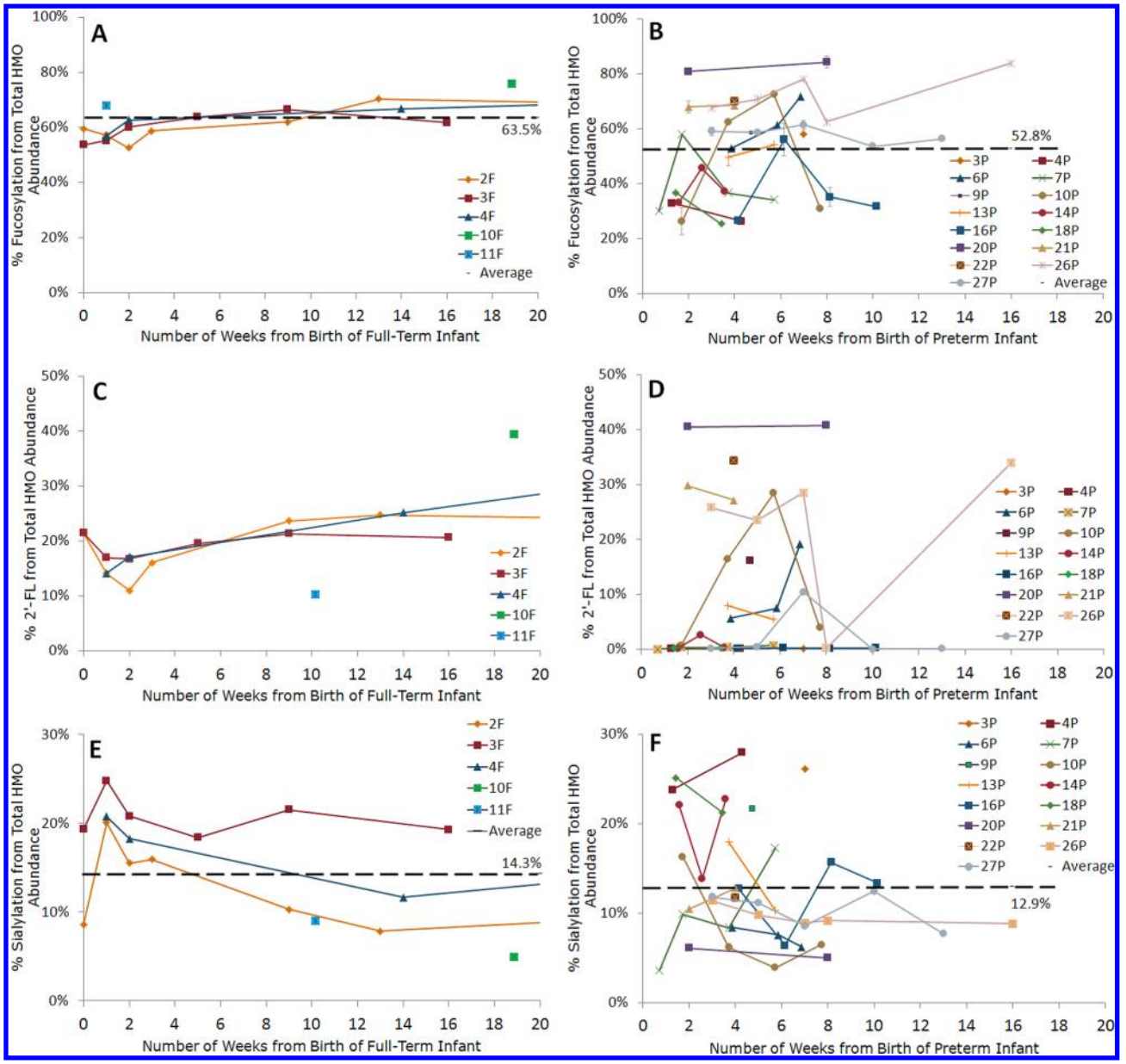

Figure 2. Line graphs of the percent fucosylation of human milk oligosaccharides (HMOs) in (A) term and (B) preterm milk during the course of lactation; percent abundance of 2′-fucosyllactose, an HMO containing an α-1,2-fucose in (C) term and (D) preterm milk; and percent sialylation of HMOs in (E) term and (F) preterm milk. Y-values are expressed as percentage from the total HMO abundance normalized per sample. Each color represents a different mother. Averages are shown as black broken lines. Average percent values for preterm milk shown in B, D, and F include all preterm milk samples (N = 41) listed in Table 1. Average percent values for term milk shown in A, C, and E include all term milk samples (N = 23) listed in Table 2.

Percentage of sialylated HMOs in milk from women delivering at term and preterm and changes in individual mothers over time are presented in Figure 2E and F. The percentage of sialylated HMOs did not differ between term and preterm milk (adjusted full-term vs preterm mean difference at 40 weeks PMA = 0.8%, 95% CI: −5.4% to 6.9%), nor was there a significant difference in variability (MADs 4.6% and 3.6% for between-mother premature and term milk and 2.4% and 2.3% for within-mother premature and term milk respectively).

Mono-, Di-, Tri-, and Tetrafucosylation in Preterm and Term Milk

Fucosylation was further investigated to determine the differences in abundance of mono, di, tri, and tetrafucosylation (based on the number of fucose residues), as shown in Tables 1 and 2. Abundance of mono, di, tri, and tetrafucosylation was similar for both preterm and full-term milk. Changes in degree of fucosylation over time for individual mothers providing multiple specimens are demonstrated in Figure 4 (preterm) and Figure 5 (term).

Secretor Status (2′-Fucosyllactose) in Preterm and Term Milk

To determine "milk secretor status", we monitored an oligosaccharide containing an α-1,2-fucose, 2′-fucosyllactose (2′-FL), with a calculated neutral mass of 490.1897 (less than 4 ppm error) and retention time of 11.720 ± 0.75 min across the lactation samples. In Figures 4 and 5, the line represents percentage of 2′-FL.

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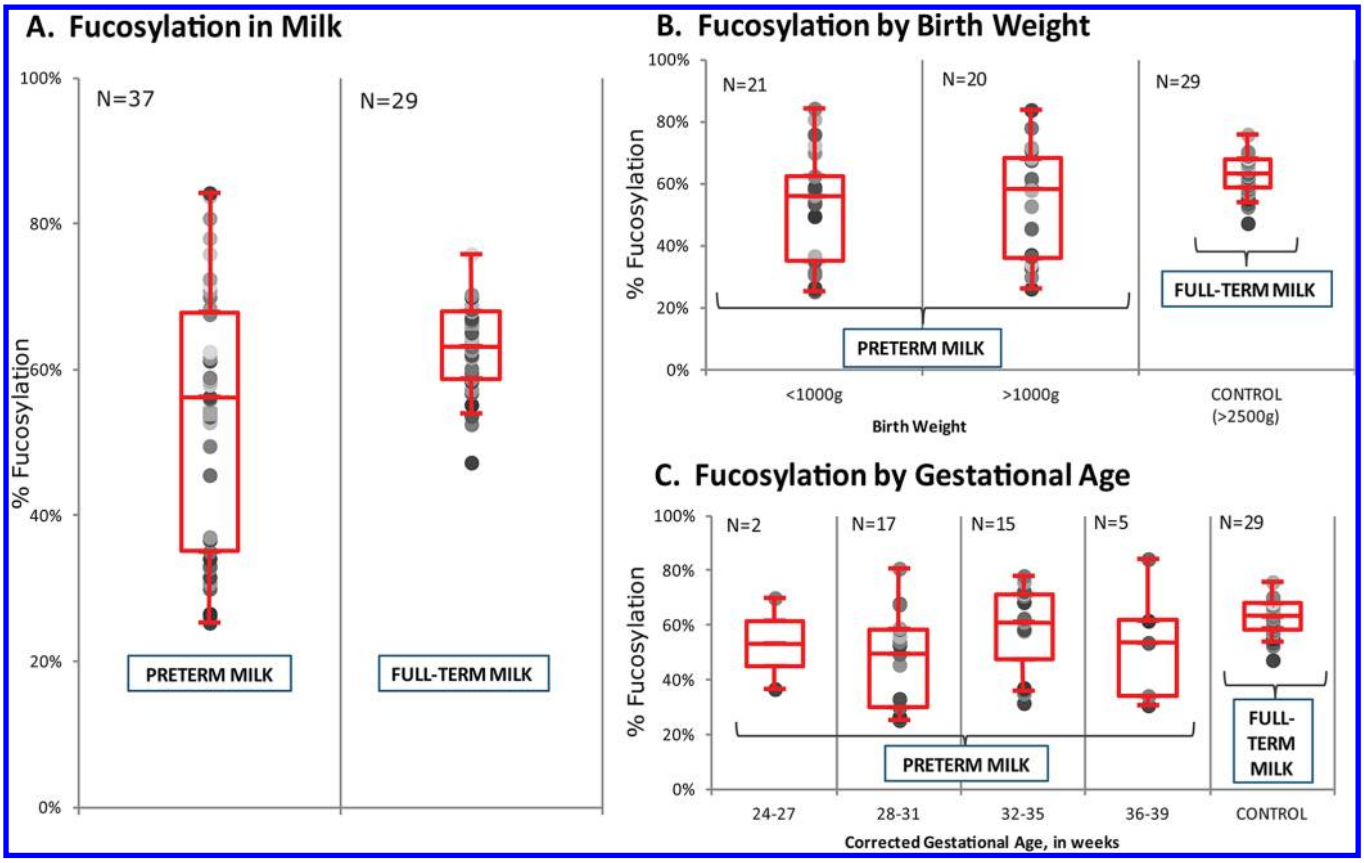

Figure 3. Fucosylation in human milk of mothers who delivered preterm and full-term infants. Preterm milk transitioning into full-term milk, that is, preterm milk collected after 36 weeks of corrected gestation were not included in the plot. Box-whisker plots of fucosylation in HMOs by (A) gestational age, (B) birth weight, and (C) corrected gestational age at the time of milk collection. *N* = number of samples.

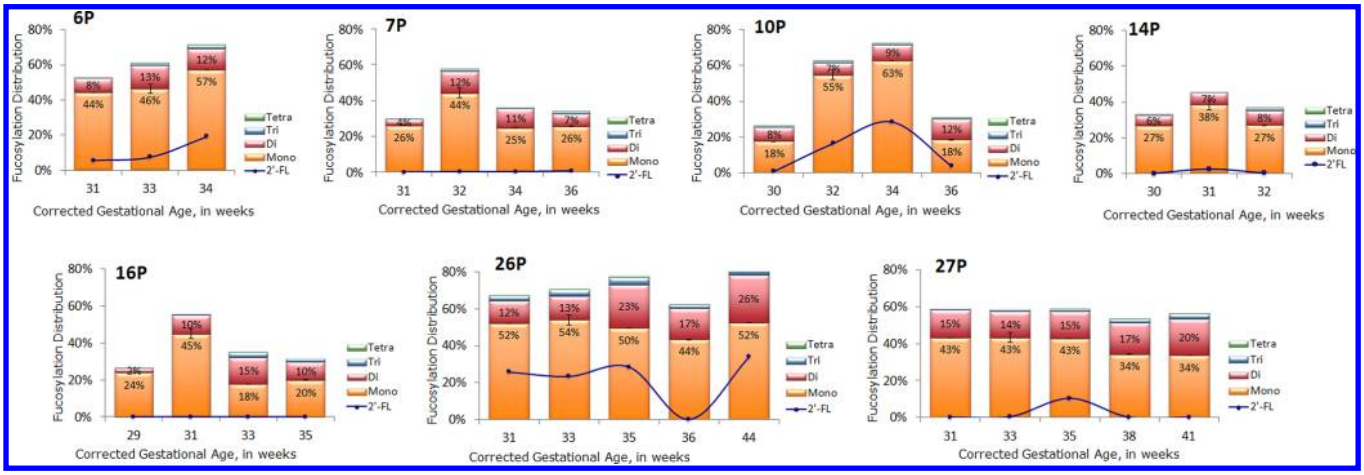

Figure 4. Bar graphs of mono, di, tri and tetrafucosylation of free HMOs in human milk of seven mothers who delivered prematurely. The line graph inset in each bar graph is the percent abundance of 2′-fucosyllactose present in the sample. Only mothers who had three or more samples were graphed. The height of each bar represents the total fucosylation in the sample.

The percentage of HMOs that were 2′-FL did not differ significantly between milk from mothers delivering preterm and term at 40 weeks PMA (adjusted term vs preterm mean difference = 0.1%, 95% CI: −10% to 12%). For 2′-FL, the between-mothers MADs were not significantly different, but the within-mothers MADs were 4.2% for the preterm group and 2.6% for the term group ($p < 0.05$), suggesting fluctuation in the secretor status of preterm milk.

Twenty-two of 23 samples from women delivering at term gave an intensity of 2′-FL greater than 5% (mean 20.5%, SD 9.7%), consistent with secretor status. One full-term mother 13F had 0.1% of 2′-FL in her free milk oligosaccharides, suggesting that she is a nonsecretor.

The preterm milk samples showed a predominance of low abundances for 2′-FL. Six of the fifteen mothers delivering preterm had a consistently low 2′-FL abundance of <5% across lactation. Another set of six mothers delivering preterm had 2′-

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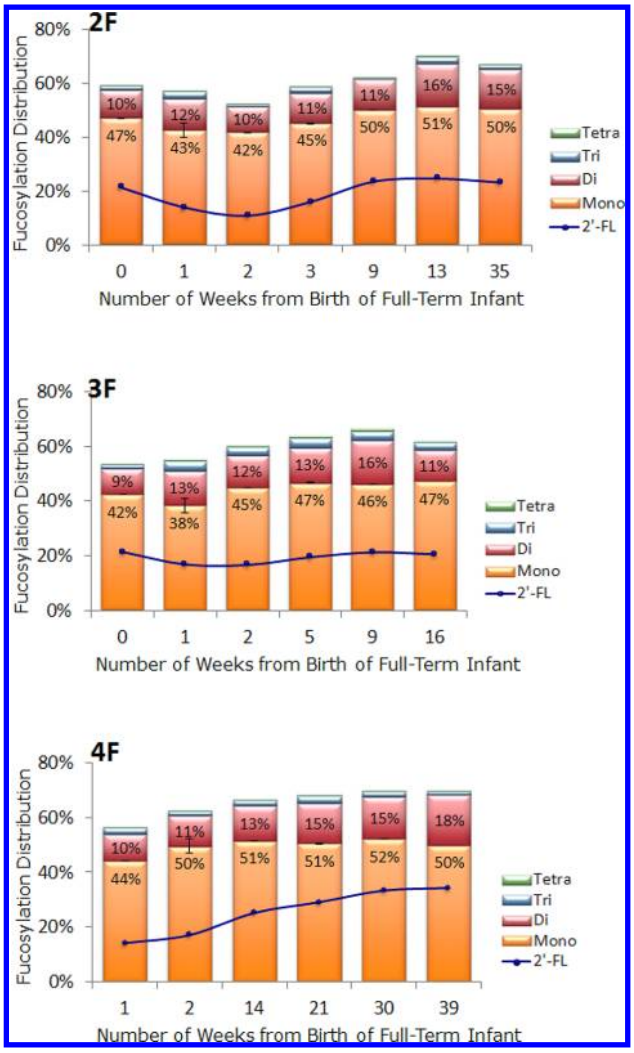

Figure 5. Bar graphs of mono, di, tri and tetrafucosylation of free HMOs in human milk of three mothers who delivered at term. The line graph inset in each bar graph is the percent abundance of 2′-fucosyllactose present in the sample. Only mothers who had three or more samples were graphed. The height of each bar represents the total fucosylation in the sample.

FL abundance greater than 5% at all time points, ranging from 5.4% to 40.7%. Three preterm mothers (10P, 26P, and 27P) had both low (<5%) and high percent abundances of 2′-FL at different time points.

The secretor status of two preterm mothers, 26P and 27P, was verified using their saliva and blood type. Mother 26P was found to be a Group O Secretor. The unexpectedly low value obtained at week 36 was reanalyzed to confirm the veracity of 0.2% abundance of 2′-FL and the same value was found. Such a low abundance in the one specimen is unexpected in a secretor mother. Mother 27P was found to be a Group AB nonsecretor. Four out of five samples gave low abundances of 2′-FL, as expected for mothers who are nonsecretors; the single elevated value was unexpected. Variation over time in individual mothers is presented in Figure 2C and D.

These results suggest that "milk secretor status" is not consistent in women delivering preterm. In contrast, 3-FL which is not influenced by secretor status, did not differ significantly between milk from mothers delivering preterm and term at 40 weeks PMA. For 3-FL, within-mothers MADs were not significantly different; the between-mother MADs were low in both groups, but differed significantly (0.38% for term and 0.04% for preterm, $p = 0.01$).

DISCUSSION

Preterm vs Term Milk

Milk from mothers delivering preterm differs from that of mothers delivering at term in content of protein,[2] fat,[36] lactose,[37] calcium,[38] and a variety of bioactive molecules.[39] Many of these differences persist as long as 8 weeks after birth, suggesting that premature delivery significantly alters lactation. We have demonstrated more abundant LNT and higher variability in LNT production in preterm milk. LNT is highly abundant in human milk and one of the primary drivers of colonization with infant strains of bifidobacteria. These data also show a higher degree of variation in percentage of fucosylated HMOs, both between women and over time, in women delivering preterm compared to women delivering full term. Premature infants are at increased risk for infections due to immaturity of many facets of innate and adaptive immunity. Given the postulated importance of fucosylated HMOs in pathogen binding, fluctuations in fucosylated HMOs in mothers' milk may further increase this risk. This high degree of variability in HMO fucosylation is further evidence of dysregulation in the "premature breast."

On the contrary, differences in the amount of between-mother and within-mother overtime variation in sialylation of HMOs between women delivering at term and preterm were not statistically significant. This differs from previous observations;[27] the small number of samples in the current study raises the possibility of a type 2 error. Given the postulated role of sialic acid in neurodevelopment,[27] further exploration of immaturity in regulation of HMO sialylation is warranted.

Secretor Status and Preterm Milk

Secretor status varies with racial and ethnic background with Japanese and western European women about 80% secretors[22] and African and Bangladeshi women about 60% secretors.[40] The presence of an α-1,2-linked fucosylated HMO, 2′-fucosyllactose (2′-FL), in human milk indicates that the mother is a secretor.[41,42] A large study of human milk as a marker of secretor status showed 100% of samples from Mexico and Sweden and 46% of samples from the Philippines suggestive of maternal secretor status.[8] The race/ethnicity of the women in this study were as follows: term 6 white non-Hispanic, 1 white Hispanic; preterm 10 white non-Hispanic, 3 white Hispanic, 2 black non-Hispanic. Our sample size is too small to confirm any racial associations with secretor status. In the present study, nano-HPLC Chip/TOF MS provided an easy method to distinguish 2′-FL from its isomer 3-fucosyllactose (bearing an α-1,3-linked fucose) since their retention times differ by at least 10 min. The unexpectedly high number of apparent nonsecretors among women delivering preterm and the lack of consistency in "milk secretor status" over time in patients 10P, 26P, and 27P support the hypothesis that fucosylation of HMOs is inconsistent in women delivering preterm. The alternative hypothesis (that nonsecretor status increases a woman's risk of delivering preterm) seems less likely given the inconsistency in the three patients noted.

The addition of fucose residues in oligosaccharides relies on the genetically determined activities of three or more distinct fucosyltransferases.[22] One of the fucosyltransferases, the α-1,2-

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fucosyltransferase, is found only in secretors, that is, people who secrete soluble blood group substances (A, B, and O(H)) that match their specific blood group type in their body fluids such as tears, milk or saliva.[41] Mothers who are nonsecretors typically do not express (or express in minute amounts) α-1,2-fucosyloligosaccharides in their milk or other body secretions.[43]

Secretor status in premature infants has recently been described as a risk factor for late onset sepsis, necrotizing enterocolitis, and death.[44] The mechanism by which secretor status is protective in this high-risk population is not clear, but the recent findings that secretor status affects the bifidobacterial composition of the intestinal microbiota in adults,[45] that premature infants generally have very low numbers of fecal bifidobacteria,[46] and that probiotic bifidobacteria appear to be protective against necrotizing enterocolitis in premature infants[47] suggest the hypothesis that nonsecretor premature infants enrich a less protective constellation of gut microbes than secretor premature infants. In term infants, milk from secretor mothers is protective against diarrhea, including that caused by campylobacter and calicivirus.[48] Nonsecretor adults have been found to be more susceptible to bacterial infections,[49,50] fungal infections,[51] and autoimmune diseases,[50] and to be protected against norovirus[52] (though the associations with norovirus gastroenteritis[53,54] and ankylosing spondylitis[55] have been questioned).

SIGNIFICANCE

Preterm infants have the disadvantages of having an immature immune system, a leaky gut, and an intestinal microbiota that differs markedly from that of the term infant.[46] They are denied the protective effects of placental transfer of maternal antibodies and of swallowed amniotic fluid that occurs mostly during the third semester of pregnancy.[56] Thus, they are prone to opportunistic infections and nutrient deficiencies at the time of maximal growth and development. Necrotizing enterocolitis is a common and devastating disease in this population; the risk of developing this disease is increased by formula feeding[57] and by an intestinal microbiota that is dominated by proteobacteria.[58,59] Given the postulated protective effects of fucosylated HMOs, hypofucosylation may increase risk of infection as well. In the case of preterm delivery, the immature breast appears unable to effectively regulate the expression of fucosylated HMOs. Correlation of degree of fucosylation with the infant's fecal microbiota would be a challenging but helpful analysis.

Donor human milk is mostly provided by women who delivered at term. Provision of pasteurized donor human milk decreases the risk of necrotizing enterocolitis compared to formula.[3,60] Pasteurization decreases human milk B cell and T cell numbers, soluble CD14, immunoglobulins, lactoferrin iron-binding activity, and lysozyme activity, but not oligosaccharide composition or quantity.[61] The observation that fucosylation in HMOs of preterm milk is highly variable raises the provocative question of whether the more consistent HMO composition of donor human milk from mothers who delivered at term may be one of the mechanisms of benefit to premature infants. Perhaps even more compelling would be to identify nonsecretor premature infants so that these infants of exceptionally high risk could be provided with highly fucosylated HMOs with the explicit goal of preventing necrotizing enterocolitis, late onset sepsis and death.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; CI, confidence intervals; ELISA, enzyme-linked immunosorbent assay; HMO, human milk oligosaccharide; LC, liquid chromatography; LNT, lacto-*N*-tetraose; MAD, mean absolute deviation; MALDI FT-ICR, matrix-assisted laser desorption/ionization Fourier transform-ion cyclotron resonance; MS, mass spectrometry; m/z, mass-to-charge ratios; PMA, postmenstrual age; TOF, time-of-flight

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