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Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices

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Abstract

Background: Human milk oligosaccharides (HMOs) shape the developing gut microbiome and influence immune function. Aside from genetic Secretor status, the factors influencing HMO synthesis and secretion are largely unknown.

Objective: We aimed to identify modifiable and nonmodifiable factors associated with HMO concentrations.

Methods: This prospective observational study included a representative subset of 427 mothers participating in the CHILD birth cohort (mean age: 33 y, 73% Caucasian). Breast milk was collected at 3–4 mo postpartum. Concentrations of 19 predominant HMOs were measured by rapid high-throughput HPLC. Secretor status was defined by the presence of 2′-fucosylactose. Associations with maternal, infant, and environmental factors were explored using multivariable regression. Breastfeeding duration was explored as a secondary outcome.

Results: Overall, 72% of mothers were Secretors and the mean ± SD duration of any breastfeeding was 12.8 ± 5.7 mo. HMO profiles were highly variable; total HMO concentrations varied 3.7-fold and individual HMOs varied 20- to >100-fold. Secretor mothers had higher total HMO concentrations than did non-Secretors (mean: 15.91 ± 2.80 compared with 8.94 ± 1.51 μmol/mL, $P < 0.001$) and all individual HMOs differed by Secretor status, except for disialyllacto-*N*-tetraose (DSLNT). Most HMO concentrations were lower in milk collected later in lactation, although some were higher including DSLNT and 3′-sialyllactose. Independent of Secretor status and lactation stage, seasonal and geographic variation was observed for several HMOs. Parity, ethnicity, and breastfeeding exclusivity also emerged as independent factors associated with some HMOs, whereas diet quality and mode of delivery did not. Together, these factors explained between 14% (for 6′-sialyllactose) and 92% (for 2′-fucosyllactose) of the observed variation in HMO concentrations. Lower concentrations of lacto-*N*-hexaose or fucodisialyllacto-*N*-hexaose were associated with earlier breastfeeding cessation.

Conclusions: HMO concentrations vary widely between mothers and are associated with multiple characteristics beyond genetic Secretor status, as well as feeding practices and environmental factors. Further research is warranted to determine how these associations affect infant health. This study was registered at clinicaltrials.gov as NCT03225534. *J Nutr* 2018;148:1733–1742.

Keywords: human milk oligosaccharides, breastfeeding, breast milk, human milk, infant, nutrition, infant feeding, environment, secretor status, FUT2

Introduction

Breastfeeding has many established benefits for maternal and child health (1), yet there is considerable variation in the associations observed across different settings and populations. This variation may be related to differences in maternal characteristics (e.g., age, parity, and genetics) and exposures (e.g., diet, environment, and lifestyle) that influence mammary gland function and milk composition. In addition to nutrients,

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human milk contains many bioactive components that support healthy infant development (2). These include probiotic bacteria (3) and prebiotic oligosaccharides (4) that drive development of the infant microbiota (5–7), a complex community of commensal microbes that contributes to host metabolism, immunity, and health across the life span (8, 9).

Human milk oligosaccharides (HMOs) are complex carbohydrates that are highly abundant in human milk, but are absent from most infant formulas (10–12). One liter of human milk contains 5–15 g of HMOs, which exceeds the concentration of all milk proteins combined (4). Although they are only minimally digested by the infant, HMOs act as selective prebiotics for the gut microbiota (13). Studies by our group and others have found associations between HMOs and epithelial cell maturation (14), infant body composition (15), and protection from necrotizing enterocolitis (16) and viral infections (17).

More than 100 HMOs have been identified, although <20 typically account for >90% of total HMO content, with the amount and composition varying substantially between women (11). Little is known about the maternal factors driving this variability, aside from the activity of galactoside 2-α-L-fucosyltransferase 2 (FUT2) and galactoside 3/4-L-fucosyltransferase (FUT3), which determine the synthesis of fucosylated HMOs (4). In a recent cross-sectional investigation, our lab profiled HMO composition in 410 healthy women from 11 international settings, finding substantial ethnic and geographic variation in FUT2 Secretor status (ranging from 65% to 98%) and HMO profiles (18). These differences support a genetic basis for HMO variation, but differences were also observed between genetically similar populations living in different locations, suggesting that sociocultural and environmental factors may also play a role. However, the specific factors contributing to HMO variability remain poorly understood.

The objective of this exploratory study was to characterize HMO profiles in a large cohort of healthy mothers, and to identify fixed, modifiable, and environmental factors associated with HMO variability. In addition, we examined the association of HMO concentrations with breastfeeding duration.

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Supplemental Tables 1–3 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: CHILD, Canadian Healthy Infant Longitudinal Development; DFLac, difucosyllactose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDR, false discovery rate; FDSLNH, fucodisialyllacto-*N*-hexaose; FUT2, galactoside 2-α-L-fucosyltransferase; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

Methods

Design and study population

This clinical study was embedded in the Canadian Healthy Infant Longitudinal Development (CHILD) Study (19) (NCT03225534). Pregnant women (n = 3407) were recruited in the general CHILD cohort between 2009 and 2012 across 4 sites in Canada (Vancouver, Edmonton, Manitoba, and Toronto). Healthy infants born at ≥35 weeks of gestation remained eligible for enrolment (n = 3264). We analyzed a representative subset (n = 427) of mother-infant dyads who provided a milk sample at 3–4 mo postpartum and had complete data on maternal diet, maternal BMI, and infant BMI (**Supplemental Figure 1**). This subset was randomly selected with equal weighting across study sites. This was a secondary analysis; the sample size was initially powered to detect a ≥10% variation in human milk leptin. This subset was representative of all eligible dyads with respect to sociodemographic and obstetric variables (**Table 1**). The primary outcome of this exploratory study was the concentration of total and individual HMOs. Breastfeeding duration was examined as a secondary outcome to explore whether HMOs might influence or serve as a biomarker of milk supply or other factors related to breastfeeding cessation. This study was approved by the Human Research Ethics Boards at McMaster University and the Universities of Manitoba, Alberta, Toronto, and British Columbia.

Breast-milk collection and HMO analysis

Mothers were instructed to express, combine, and refrigerate samples of foremilk and hindmilk from multiple feeds during the 24 h before the CHILD Study home visit at 3–4 mo postpartum (median: 16 wk, IQR: 14–19 wk) (20). Hand expression was encouraged, although pump expression was also accepted. Samples were stored at −80°C until analysis. As previously described (18), raffinose was added to each sample as an internal standard for absolute quantification. HMOs were isolated by high-throughput solid-phase extraction, fluorescently labeled, and analyzed by HPLC with fluorescence detection. Nineteen HMOs (listed in **Figure 1**) were quantified based on standard retention times and mass spectrometric analysis. These 19 HMOs typically account for >90% of total HMO content; their concentrations were summed to estimate total HMO concentration. The relative abundance of each HMO was also calculated. FUT2 "Secretor status" was defined by the presence or near absence of 2'-FL. Total HMO-bound fucose and total HMO-bound sialic acid were calculated. To assess the overall diversity of HMO composition, Simpson's Diversity index was calculated as the reciprocal sum of the square of the relative abundance of each HMO.

Exposure variables

In this exploratory analysis, we assessed a variety of factors that could plausibly influence HMO composition, based on previous research and hypothesized mechanisms of HMO synthesis and secretion. Maternal age, ethnicity, education (postsecondary degree), smoking, parity, and multivitamin use were self-reported during pregnancy. A validated FFQ (21, 22) was administered during late pregnancy to capture usual dietary intake during this pregnancy, and used to estimate total energy intake (kilocalories per day) and the Healthy Eating Index score (23). Maternal prepregnancy BMI was derived from measured height and self-reported prepregnancy weight, validated against health records (24). Maternal overweight and obesity were defined as BMI (kg/m^2) >25.0 and >30.0, respectively. The nurse at delivery recorded infant sex, gestational age, birth weight, and mode of delivery. Infant feeding practices, including age at weaning and introduction of formula or complementary foods, were reported by mothers by standardized questionnaire at 3, 6, 12, 18, and 24 mo (25) and used to determine total breastfeeding duration and breastfeeding status at the time of sample collection: exclusive (breast milk only) or partial (breast milk supplemented with formula, solid foods, or both). Lactation stage (weeks postpartum) and season (calendar month) were also documented.

Statistical analysis

All statistical analyses were performed using R (version 3.3.2; R Foundation for Statistical Computing). To address potential selection

TABLE 1 Characteristics of the study population compared with all eligible dyads in the CHILD cohort[1]

	Subset for this analysis ($n = 427$)	All eligible[2] dyads ($n = 2536$)
Mother		
Age, y	33.0 ± 4.2	32.7 ± 4.2
Diet quality, HEI-2010 score	74.0 ± 8.3	73.7 ± 8.3
Prenatal multivitamin intake	280 (71.6)	281 (71.5)
Site		
Edmonton	107 (25.1)	566 (22.3)
Toronto	107 (25.1)	580 (22.9)
Vancouver	107 (25.1)	646 (25.5)
Winnipeg	106 (24.8)	744 (29.3)
Ethnicity		
Asian	78 (18.3)	403 (16.0)
Caucasian	314 (73.5)	1870 (74.3)
First Nations	17 (4.0)	87 (3.5)
Other	18 (4.2)	158 (6.3)
Prepregnancy BMI, kg/m^2	24.7 ± 6.4	24.5 ± 6.3
Normal: <25	279 (65.3)	1598 (66.9)
Overweight: ≥25–30	88 (20.6)	509 (21.3)
Obese: >30	60 (14.1)	282 (11.8)
Gestational weight gain		
Inadequate	38 (14.3)	242 (14.5)
Adequate	104 (39.2)	685 (40.9)
Excessive	123 (46.4)	747 (44.6)
Parity		
0	234 (54.8)	1345 (53.1)
1	136 (31.9)	860 (33.9)
≥2	57 (13.3)	330 (13.0)
Postsecondary degree	352 (83.0)	1979 (80.3)
Infant		
Sex, male	220 (51.5)	1338 (52.8)
Gestational age at birth, wk	39.2 ± 1.2	39.2 ± 1.2
Birthweight, g	3470 ± 477	3450 ± 479
Method of birth		
Elective cesarean	48 (11.4)	264 (10.6)
Emergency cesarean	52 (12.4)	339 (13.6)
Vaginal	321 (76.2)	1893 (75.8)
Milk sample and breastfeeding		
Lactation stage, wk postpartum	17.1 ± 5.0	16.5 ± 5.0
Season of sample collection		
Spring (March–May)	82 (19.2)	604 (23.9)
Summer (June–August)	113 (26.5)	698 (27.6)
Fall (September–November)	121 (28.4)	623 (24.6)
Winter (December–February)	110 (25.8)	606 (23.9)
Method of sample collection		
Hand expression	25 (5.9)	203 (8.7)
Pumped	101 (23.7)	575 (24.8)
Unknown	301 (70.5)	1545 (66.5)
Exclusive BF at time of sample	213 (51.2)	1393 (55.9)
Duration of exclusive BF, mo	3.6 ± 2.3	3.7 ± 2.3
Duration of any BF, mo	12.8 ± 5.7	12.5 ± 5.6
FUT2 secretor status, positive	307 (71.9)	Unknown

[1] Values are *n* (%) or means ± SDs. Percentages reflect proportion of nonmissing data. BF, breastfeeding; CHILD, Canadian Healthy Infant Longitudinal Development; FUT2, 2-α-L-fucosyltransferase 2; HEI, Healthy Eating Index.
[2] Those who breastfed ≥12 wk and provided a milk sample.

bias, characteristics of dyads included in this analysis were compared with all eligible dyads (those breastfed for >12 wk) via *t* tests and chi-square tests (Table 1). HMO profiles were visualized using *ggplot* and summarized using descriptive statistics. Nonparametric Spearman rank correlation, Mann-Whitney, and Kruskal-Wallis tests were used to assess bivariate associations between HMOs (non-normally distributed), Secretor status, and other exposure variables; associations were visualized using heat maps with hierarchic clustering (*ComplexHeatmap*), correlograms (*ggcorrplot*), boxplots (*ggplot2*), and scatterplots with linear or polynomial fits (*ggplot2*). Multivariable linear regression was used to address potential confounding bias and identify factors independently associated with HMO concentrations and diversity (dependent variables; log-transformed and converted to *z* scores). Candidate factors (independent variables) were selected a priori (maternal age, Secretor status, lactation time, infant sex, and method of birth) or identified through bivariate analyses (all variables demonstrating a significant association ($P < 0.05$) with any HMO were included in all models). Regression results [adjusted β estimates ($a\beta$) and *P* values] were summarized visually with a correlogram. The association of HMO concentrations (as independent predictor variables) and breastfeeding duration (as the dependent outcome variable) was determined using linear regression, adjusting for potential confounders (lactation time postpartum and breastfeeding exclusivity at sample collection, ethnicity, parity, and study site) and known predictors of breastfeeding duration identified from the literature (maternal BMI, education, and diet quality as a proxy for lifestyle). Dyads with missing data for exposure variables included in the multivariable model were excluded. False discovery rate (FDR) correction was applied to adjust for multiple comparisons.

Results

The mean ± SD maternal age was 33.0 ± 4.2 y; the majority of mothers were Caucasian (73%) and had a postsecondary degree (83%) (Table 1). Fifty-five percent were first-time mothers and 24% delivered by cesarean delivery. The mean ± SD lactation stage was 17.1 ± 5.0 wk postpartum; over half (52%) of mothers were exclusively breastfeeding at this time, whereas the remainder were supplementing with formula (27%), solid foods (12%), or both (8%). Total HMO concentrations ranged from 5.8 to 21.4 μmol/mL (mean ± SD: 14.0 ± 4.0). The mean ± SD duration of any breastfeeding was 12.8 ± 5.7 mo.

HMO profiles

Overall, 307 (72%) mothers were Secretors and 120 (28%) were non-Secretors. Caucasian mothers were more likely to be Secretors than Asian mothers (74% and 60%, respectively, $P = 0.04$) (**Supplemental Table 1**). As expected, non-Secretor mothers produced significantly less HMOs (mean ± SD: 8.94 ± 1.51 compared with 15.90 ± 2.80 μmol/mL, $P < 0.001$) and this difference was primarily due to the absence of 2'-fucosyllactose (2'FL), which accounted for 38.6% ± 14.6% of total HMOs in Secretor milk (Figure 1, **Supplemental Tables 2 and 3**). Within Secretor groups, HMO profiles were highly variable between mothers, in terms of total HMO content (range: 7.68–21.40 μmol/L among Secretors and 5.80–13.70 μmol/L among non-Secretors) and relative HMO composition [e.g., 1.2–74.3% 2'FL among Secretors; 6.0–56.2% lacto-*N*-tetrose (LNT) among non-Secretors].

In addition to expected differences in FUT2-dependent HMOs [2'FL, difucosyllactose (DFLac), and lacto-*N*-fucopentaose (LNFP) I], most other HMOs also differed significantly by Secretor status, with the exception of 6'-sialyllactose (6'SL) and disialyllacto-*N*-tetraose (DSLNT) (Figure 1C). Some were present at higher concentrations in

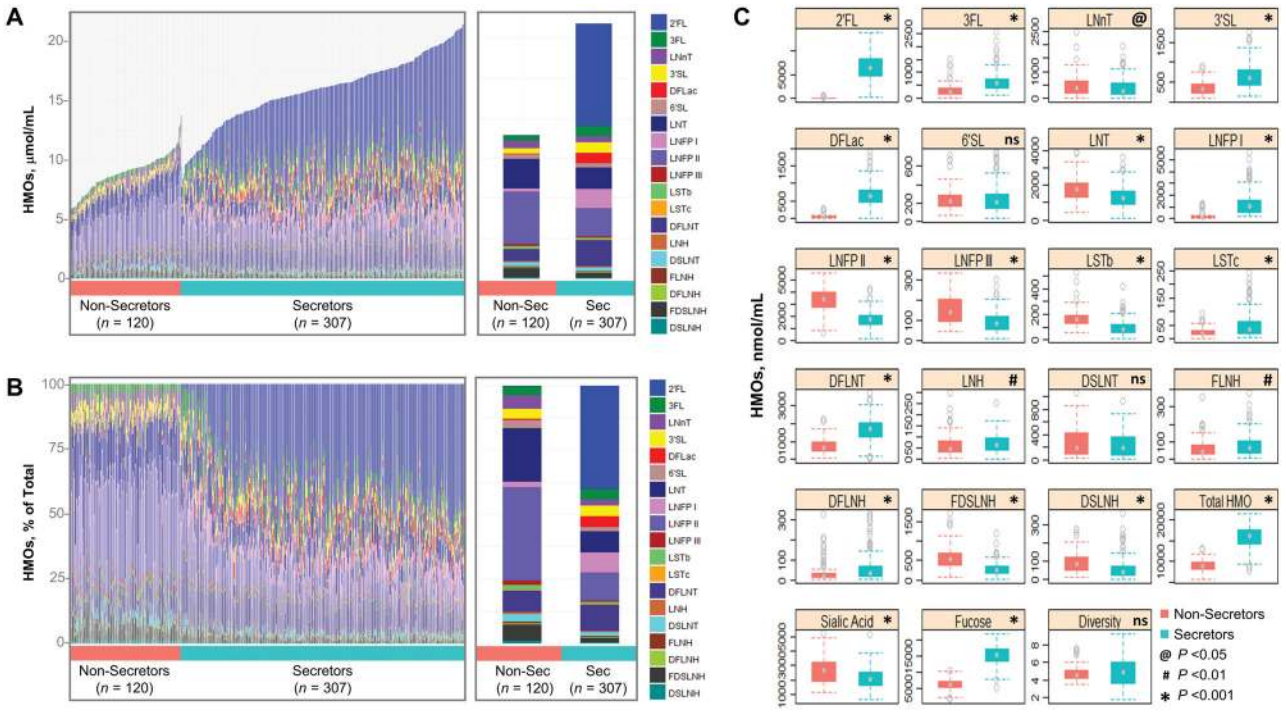

FIGURE 1 HMO concentrations (A, C) and relative composition (B) among 427 Canadian mothers in the CHILD cohort, overall (A, B) and by Secretor status (C). (A) Absolute HMO concentrations (nmol/mL) and (B) relative HMO composition (percentage of total HMO) for individual mothers sorted by Secretor status and total HMO concentration, and mean values by Secretor status. (C) HMO concentrations by Secretor status, compared by Mann-Whitney test with false discovery rate adjustment for multiple comparisons. ns, $P \geq 0.05$; $^{@}P < 0.05$; $^{\#}P < 0.01$; $^{*}P < 0.001$. Boxes indicate IQRs; grey dots indicate medians; whiskers indicate ranges (IQRs), excluding outliers; open circles indicate outliers: values below (–1.5*IQR), or > (+1.5*IQR). CHILD, Canadian Healthy Infant Longitudinal Development; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDSLNH, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; Fucose, human milk oligosaccharide–bound fucose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; Non-Sec, non-Secretors; Sec, Secretors; Sialic Acid, HMO-bound Sialic Acid; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

Secretor milk [3-fucosyllactose (3FL), 3'-sialyllactose (3'SL), difucosyllacto-*N*-tetrose (DFLNT), and sialyl-lacto-*N*-tetraose c (LSTc)], whereas others were enriched in non-Secretor milk [LNFP II, LNFP III, LNT, sialyl-lacto-*N*-tetraose (LSTb), fucodisialyllacto-*N*-hexaose (FDSLNH), and disialyllacto-*N*-hexaose (DSLNH)] (all $P < 0.001$).

Clustering and correlation between HMOs

As expected, FUT2-dependent HMOs (2'FL, DFLac, and LNFP I) clustered together and their concentrations were positively correlated (Spearman $\rho = +0.37$ to $+0.70$) (Figure 2A). These and other HMOs enriched in Secretor milk (i.e., 3FL, 3'SL, and DFLNT) were negatively correlated with HMOs enriched in non-Secretor milk (LNFP II, LNFP III, LNT, LSTb, and FDSLNH). 6'SL and DSLNT, the 2 HMOs that were not associated with Secretor status, clustered together and did not correlate with any other HMOs. Some distinct correlations emerged after stratifying by Secretor status (Figure 2B, C); for example, in non-Secretor milk, DSLNT was negatively associated with 3FL ($\rho = -0.56$) and positively associated with 3'SL ($\rho = +0.62$), whereas neither correlation was observed in Secretor milk ($\rho = 0.04$ and -0.03, respectively).

Univariate analyses of HMOs by stage of lactation and season of collection

Total HMO concentration and diversity were relatively stable across the lactation period captured in our study (6–39 wk postpartum); however, we observed significant differences for several individual HMOs (Figure 3A). Regardless of Secretor status, the following HMOs were lower in milk collected later in lactation: lacto-*N*-hexaose (LNH), difucosyllacto-*N*-hexaose ($\rho = -0.21$ and -0.25, respectively), and especially LSTc and fucosyllacto-*N*-hexaose ($\rho = -0.32$ and -0.34, respectively). In contrast, 2 HMOs had consistently higher concentrations later in lactation: 3'SL ($\rho = +0.17$) and DSLNT ($\rho = +0.28$). Among Secretors only, 6'SL, LNFP I, and DSLNH concentrations were lower later in lactation, whereas 3FL, DFLac, and LNFP III concentrations were higher. Other HMOs were not correlated with lactation time (e.g., LNFP II, DFLNT, FDSLNH). Most HMOs did not obviously differ by season of collection, with 1 exception: LNFP III concentrations were significantly lower in milk collected during winter or spring compared with summer or fall (Figure 3B).

Univariate analysis of HMOs and maternal diet

Total HEI score, reflecting overall diet quality, was not correlated with HMO concentrations (**Supplemental Figure 2**). Total HMO concentration was not correlated with any individual HEI component, although a few components were associated with individual HMOs. Fucosyllacto-*N*-hexaose was positively correlated with whole grains and LSTb was negatively correlated with total protein and empty calories. LNT and difucosyllacto-*N*-hexaose were positively correlated with total energy intake. However, these associations were relatively weak

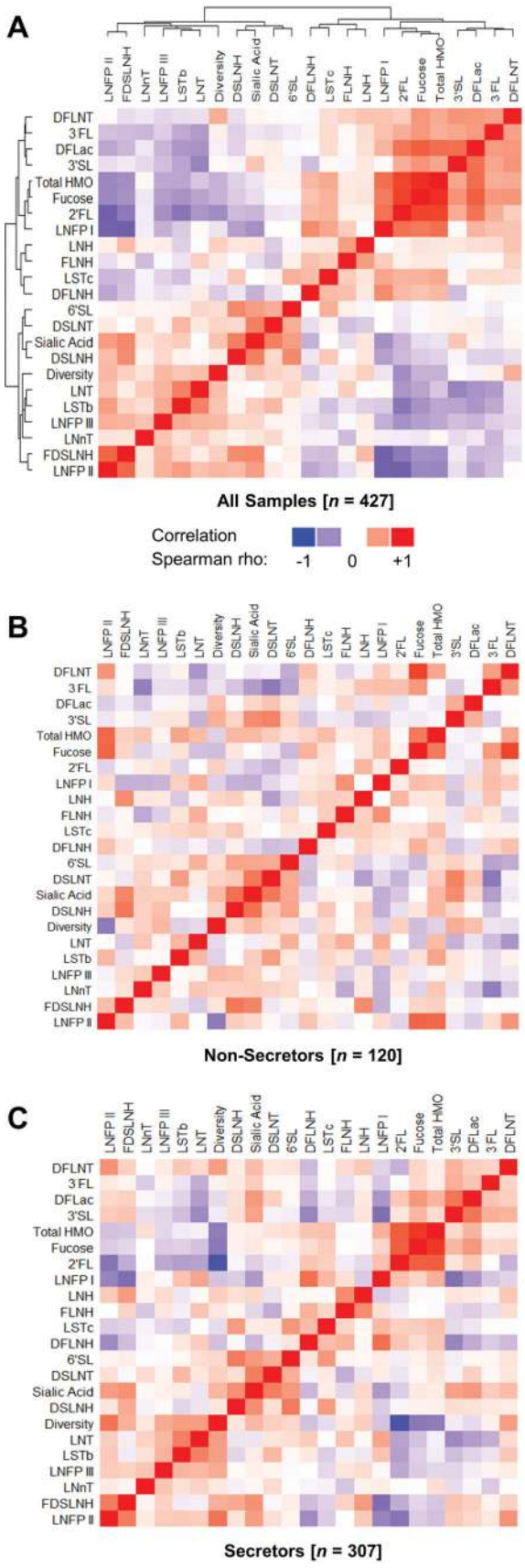

FIGURE 2 Correlation between HMO concentrations among 427 Canadian mothers in the CHILD cohort, overall (A) and by Secretor status (B, C). HMOs were clustered according to their correlation pattern among all samples; the same order was applied in analyses stratified by Secretor status. Color and shading reflect direction and strength of Spearman rank correlation coefficients (blue = negative; red = positive; white = no correlation; darker = stronger correlation). CHILD, Canadian Healthy Infant Longitudinal Development; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDSLNH, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; Fucose, HMO-bound fucose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; Sialic Acid, HMO-bound Sialic Acid; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

(range: $\rho = -0.11$ to $+0.15$) and were nonsignificant after FDR correction.

Multivariable analysis: factors associated with HMO composition

In multivariable models predicting HMO concentration *z* scores (Figure 4), positive Secretor status (a$\beta = +1.81$ *z* score; 95% CI: 1.67, 1.94 *z* score) and lactation stage (a$\beta = -0.09$/mo; 95% CI: −0.17, −0.004/mo) were the only factors independently associated with total HMO concentration. However, in addition to these 2 factors, several other nonmodifiable, modifiable, and environmental factors were associated with individual HMO concentrations.

Nonmodifiable factors. Secretor status was strongly associated with most individual HMO concentrations, with adjusted estimates ranging from −1.24 (LNFP II) to +2.12 (2′FL). DSLNT was the only HMO not associated with Secretor status in adjusted models. About half of individual HMOs (9/19) were significantly associated with lactation stage in adjusted models, including DSLNT (a$\beta = +0.23$/mo; 95% CI: 0.10, 0.36/mo) and LSTc (a$\beta = -0.24$/mo; 95% CI: −0.37, −0.12/mo). Other significant nonmodifiable factors included maternal age (lower DFLNT concentrations in older mothers), parity [higher lacto-*N*-neotetraose (LNnT) and LNT, and lower 3FL in multiparous mothers], and ethnicity (higher LSTc and lower FDSLNH in Asian compared with Caucasian mothers).

Modifiable factors. Few of the modifiable factors examined were associated with HMO concentrations. LNH was lower in obese mothers and DSLNH was higher in those taking multivitamins. Solid food introduction was associated with higher LNnT and lower 3FL, whereas formula supplementation was associated with higher 6′SL and LSTb. Maternal diet quality and method of delivery were not associated with HMO concentrations.

Environmental factors. Independent of the above factors, some HMOs differed by study site (e.g., compared with Toronto: higher LNT in Edmonton and Vancouver; lower DFLNT in Edmonton and Winnipeg; lower LNnT in Vancouver). In addition, the seasonal differences observed in LNFP III (lower in spring) were confirmed in adjusted models, and several other seasonal patterns emerged (e.g., higher LNnT and lower 6′SL in winter; higher DSLNT in spring).

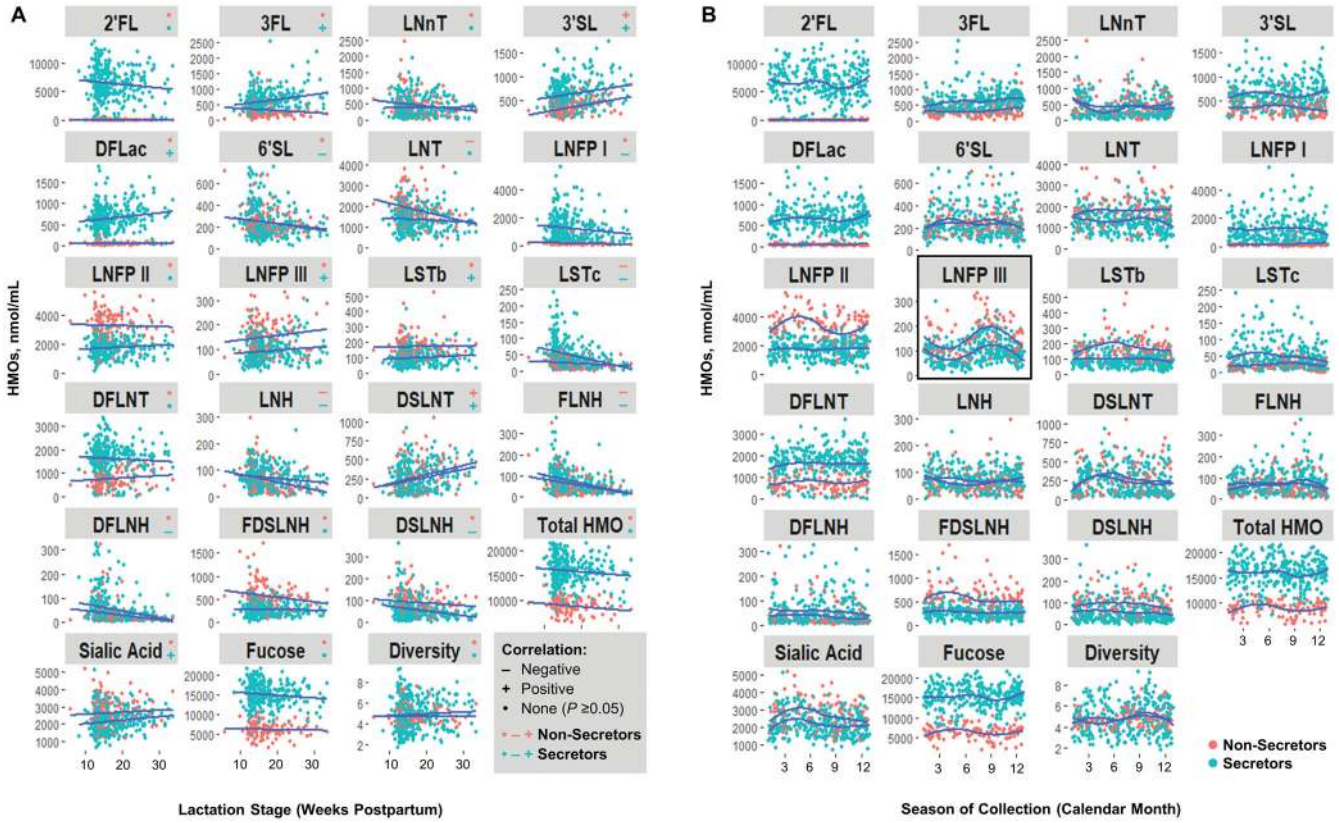

FIGURE 3 Correlation of HMO concentrations with lactation time postpartum (A) and calendar month of sample collection (B) among 427 Canadian mothers in the CHILD cohort, by Secretor status. Each dot represents 1 sample. Colors indicate Secretor status (pink = non-Secretor, blue = Secretor). Lines (A) and curves (B) indicate linear and polynomial fits, respectively, separately for non-Secretors and Secretors. (A) Some HMO concentrations are significantly lower (–) or higher (+) in milk collected later in lactation; *P* value thresholds were applied after false discovery rate correction for multiple comparisons. (B) Most HMOs are not associated with calendar month of collection, but LNFP III concentrations peak in the fall (month 10). CHILD, Canadian Healthy Infant Longitudinal Development; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDSLNH, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; Fucose, HMO-bound fucose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; Sialic Acid, HMO-bound Sialic Acid; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

Together, these nonmodifiable, modifiable, and environmental factors explained 70% of the observed variation in total HMO concentrations. The variation explained for individual HMOs ranged from 14% (LNnT) to 92% (2′FL) and was generally higher for FUT2-dependent HMOs. Associations with study site, maternal age, and BMI were not significant after FDR correction. The following variables were not associated with HMO concentrations in univariate analyses (not shown) and were therefore not included in multivariable models: smoking, infant birth weight, and gestational age (range examined: 36–41 wk).

HMOs and breastfeeding duration

Breastfeeding duration did not differ by Secretor status (13.0 mo among non-Secretors compared with 12.8 mo among Secretors); however, several HMOs were significantly associated with breastfeeding duration (Figure 5). Each *z* score increase in LNH was associated with a 1-mo increase in breastfeeding duration (aβ = +0.96 mo; 95% CI: 0.40, 1.52 mo), independent of lactation time and breastfeeding exclusivity at milk sample collection, and other potential confounders. Higher FDSLNH was also associated with longer breastfeeding duration (aβ = +0.64 mo; 95% CI: 0.13, 1.15 mo) whereas an inverse association was found for 3′SL (aβ = −0.54 mo; 95% CI: −1.07, −0.02 mo).

Discussion

Our results from the general population–based CHILD cohort show that HMO content and composition are highly variable between mothers, and identify several fixed and modifiable factors associated with this variation. Total HMO content varied by 3.7-fold and individual HMO concentrations varied by 20- to >100-fold. Although this variation was strongly associated with Secretor status, considerable variation was also observed *within* Secretor groups (2–3-fold difference in total HMO; 8- to >100-fold difference in individual HMOs), indicating that other factors play a significant role in determining HMO content and composition. Indeed, we found significant and independent associations between HMO composition and lactation stage, parity, ethnicity, city of residence, season of milk collection, and breastfeeding exclusivity. Aside from Secretor status, most factors were associated with only 1 or a few individual HMOs, suggesting that distinct mechanistic pathways influence the synthesis of different HMOs.

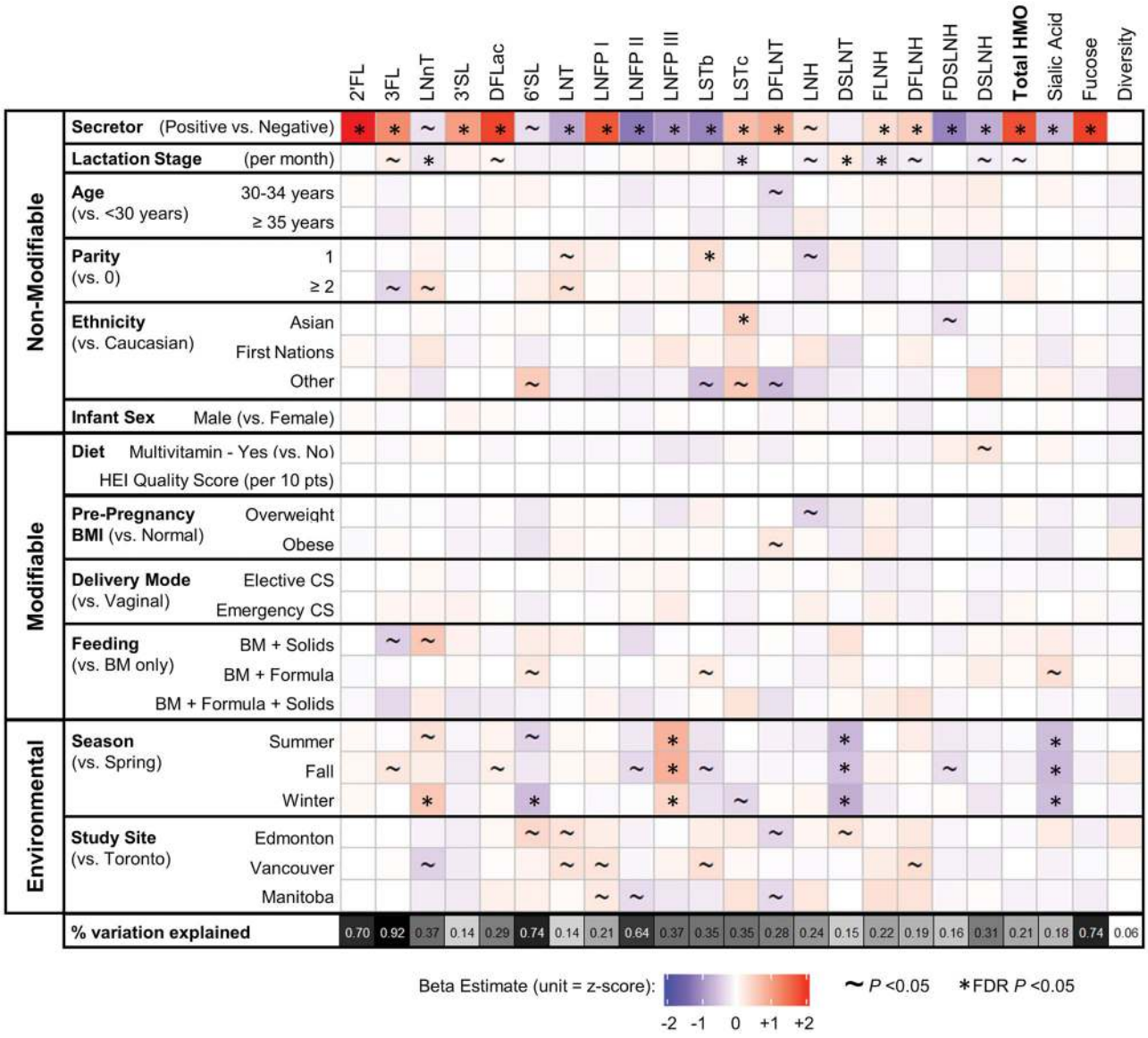

FIGURE 4 Summary of multivariable analysis of modifiable, nonmodifiable, and environmental factors and HMO composition among 427 Canadian mothers in the CHILD cohort. Beta estimates are from multivariable linear regression models for each HMO (absolute concentrations were log-transformed, then converted to *z* scores). Coloring reflects direction and magnitude of effect estimate. $^{\sim}P < 0.05$, $^{*}P < 0.05$ after false discovery rate adjustment for multiple comparisons. Predictor variables were selected a priori (maternal age, Secretor status, lactation stage, method of delivery, infant sex) or based on univariate analyses. Other variables considered in univariate analyses, showing no significant associations with HMOs, included: maternal smoking, infant birth weight, and infant gestational age (range 36–41 wk). BM, breast milk; CHILD, Canadian Healthy Infant Longitudinal Development; CS, Cesarean Section delivery; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDR, false discovery rate; FDSLNH, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; Fucose, HMO-bound fucose; HEI, Healthy Eating Index; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; Sialic Acid, HMO-bound Sialic Acid; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

Our study contributes new information about HMO composition in healthy mothers. The total HMO content and variation in our cohort (mean ± SD: 13.7 ± 4.0 μmol/mL) were comparable to the 11 countries reported by McGuire et al. (18) (range: 9.7 ± 3.9 μmol/mL in rural Ethiopia to 16.0 ± 4.9 μmol/mL in Sweden). In our multiethnic Canadian sample, 74% of Caucasian mothers were Secretors (comparable with the European sites reported by McGuire et al.), compared with just 60% of Asian mothers. This ethnic variation likely reflects evolutionary pressures for or against α1–2-fucoslylated HMOs, which may have structure-specific effects on infant development and disease risk (26). Interestingly, in our multivariable analysis, some HMOs differed by ethnicity even after adjusting for Secretor status, suggesting that other genetic or sociodemographic factors associated with ethnicity may contribute to HMO composition.

Our results are consistent with previous research showing that total HMO content declines over the course of lactation (18, 27, 28); however, our analysis reveals that some individual HMOs do not follow this trend. In particular, DSLNT and 3'SL concentrations were consistently higher in milk collected later in lactation. This may reflect a unique role for these HMOs later in infancy as requirements for growth, immunity,

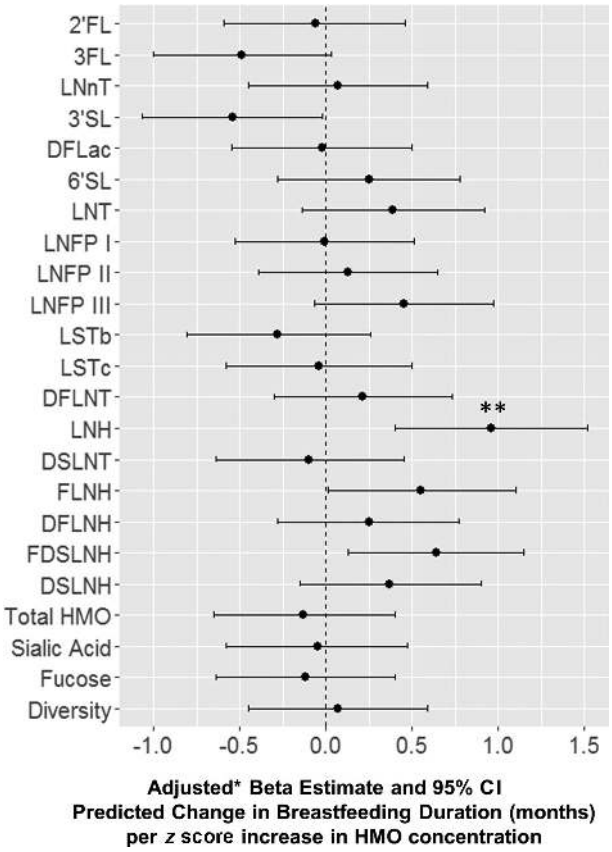

FIGURE 5 HMO composition and breastfeeding duration in the CHILD cohort. HMOs were log-transformed and converted to *z* scores. Effect estimates reflect the change in duration of any breastfeeding (in mo) for each *z* score increase in HMO concentration. Lines indicate 95% CIs from linear regression models. *Adjusted for lactation time (weeks postpartum), ethnicity, parity, education, BMI, diet quality (Healthy Eating Index score), study site, and breastfeeding exclusivity at milk sample collection. $^{**}P < 0.05$ after false discovery rate correction for multiple comparison. HMO, human milk oligosaccharide. CHILD, Canadian Healthy Income Longitudinal Development; DFLac, difucosyllactose; DFLNH, difucosyllacto-*N*-hexaose; DFLNT, difucosyllacto-*N*-tetrose; DSLNH, disialyllacto-*N*-hexaose; DSLNT, disialyllacto-*N*-tetraose; FDSLNH, fucodisialyllacto-*N*-hexaose; FLNH, fucosyllacto-*N*-hexaose; Fucose, HMO-bound fucose; HMO, human milk oligosaccharide; LNFP I/II/III, lacto-*N*-fucopentaose-I/II/III; LNH, lacto-*N*-hexaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetrose; LSTb/c, sialyl-lacto-*N*-tetraose b/c; Sialic Acid, HMO-bound Sialic Acid; 2′FL, 2′-fucosyllactose; 3FL, 3-fucosyllactose; 3′SL, 3′-sialyllactose; 6′SL, 6′-sialyllactose.

gut microbiota, and neurodevelopment are reprioritized by the developing infant. Other studies have reported contrasting results for DSLNT, showing negative correlations with lactation time (18, 29), although they assessed an earlier period of lactation (first 3 mo) than did our study (2–7 mo). Another study found no change in 3′SL over the first 4 mo of lactation (27) but did not test HMO concentrations later in lactation.

Notably, HMO differences by lactation stage were not explained by declining breastfeeding exclusivity, which was independently associated with HMO composition. For example, whereas LNnT concentrations were generally lower later in lactation, this HMO was enriched after the introduction of solid foods. The opposite pattern was observed for 3FL, which appeared to increase over lactation, but was depleted after the introduction of solid foods. Interestingly, formula supplementation did not appear to affect these 2 HMOs, although others (6′SL and LSTb) were enriched in mothers providing formula to their infants. These associations might reflect changes in HMO synthesis or secretion according to the volume of milk production, the permeability of tight junctions in the mammary gland, or the levels of other milk components, all of which are known to change over the course of lactation and with the frequency of milk removal or introduction of complementary foods (2, 30).

Another novel finding is the subtle but significant seasonal variation in some HMOs (e.g., higher LNFP III in fall and, in multivariable analyses, higher LNnT in winter and higher DSLNT and 6′SL in spring). An Australian study reported major increases in cow milk oligosaccharide concentrations during the 8-mo milking season (from spring through fall) (31); however, because all calves are born at the same time of year, it is unclear whether these differences reflect the normal progression of lactation or true "seasonal" changes. A study of 33 Gambian women (32) found that mothers produced more HMOs in the dry season (when food is more plentiful), although individual HMOs were not examined. The authors speculated that higher energy intake in the dry season may be responsible for higher HMO synthesis, but this explanation is unlikely in our Canadian population. Our findings therefore suggest that other seasonal factors in Canada (e.g., climate, sunlight, or allergen exposures) might influence HMO synthesis.

Our multivariable analyses also identified potential differences in HMO composition across the 4 study sites, independent of the aforementioned sociodemographic variables. Because all sites followed a standardized protocol for milk collection and processing, and all samples were analyzed together, this suggests a possible role for other unmeasured geographic or site-specific factors (e.g., climate, lifestyle, and outdoor or indoor environments).

To date, few studies have examined the effect of maternal diet on HMO composition. One study found that vitamin A intake was positively associated with sialylated HMOs (33), whereas another found no effect of a lipid-based nutrient supplement (34). We found no strong evidence of dietary influence on HMO composition using an index measure of diet quality during pregnancy; however, a more detailed assessment of nutrient intake during lactation may be required to identify (or exclude) dietary effects on HMO composition.

Finally, parity was independently associated with some HMOs; a finding that has not been previously reported, to our knowledge. Mode of delivery, infant sex, and (with a few exceptions) maternal age and prepregnancy BMI were largely unassociated with HMO composition.

Interestingly, lower concentrations of LNH or FDSLNH were associated with earlier weaning, independent of known predictors of breastfeeding duration. This could reflect associations with milk supply or mastitis—either causal or consequential—however, these conditions were not documented in our cohort.

The major strengths of this study are the quantitative method of HMO analysis, large sample of healthy mothers across different geographic settings, and multivariable analysis to establish independent associations of multiple fixed, modifiable, and environmental factors with HMO composition. It is possible that some associations arose by chance because of multiple testing, although we applied FDR correction to minimize this risk in our exploratory analysis. Another limitation is that we did not collect a full breast expression or standardize the timing of milk collection, which may have

influenced HMO variability. Also, although pooling of breastmilk samples across a 24-h period provided an estimate of average HMO composition, it precluded analysis of potential diurnal fluctuations. Finally, because we collected only 1 sample per mother, we could not examine HMO changes over time; however, we detected patterns across the stages of lactation captured in our large multiethnic cohort. Further research will be required to determine if our findings in the Canadian CHILD cohort are generalizable to other settings and populations.

In summary, this study provides novel information about "normal" variation in HMO composition, and identifies several fixed and modifiable factors contributing to this variation. Understanding the factors that determine HMO composition has important clinical implications because HMOs have immediate and long-term effects on gut microbiota development, infant health, and disease risk.

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