Pediatric RESEARCH

POPULATION STUDY ARTICLE

Human milk oligosaccharides, infant growth, and adiposity over the first 4 months of lactation

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BACKGROUND: The relationship between human milk oligosaccharides (HMOs) and infant growth and adiposity is not fully understood and comprehensive studies are missing from the current literature.
METHODS: We screened and recruited 370 healthy, pregnant women and their infants from seven European countries. Breastmilk samples were collected using standardized procedures at six time points over 4 months, as were infant parameters. Correlations and associations between HMO area under the curve, anthropometric data, and fat mass at 4 months were tested.
RESULTS: Lacto-N-neotetraose had a negative correlation with the change in length ($rs = -0.18$, $P = 0.02$). Sialyllacto-N-tetraose c (LSTc) had a positive correlation with weight for length ($rs = 0.19$, $P = 0.015$). Infants at the 25th upper percentile were fed milk higher in 3′-sialyllactose and LSTc ($P = 0.017$ and $P = 0.006$, respectively) compared to the lower 25th percentile of the weight-for-length *z*-score gain over 4 months of lactation. No significant associations between growth and body composition and Lewis or secretor-dependent HMOs like 2′-fucosyllactose were identified.
CONCLUSIONS: Changes in the HMO composition of breastmilk during the first 4 months appear to have little influence on infant growth and body composition in this cohort of healthy mothers and infants.

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IMPACT:

- Modest associations exist between individual HMO and infant growth outcomes at least in healthy growing populations.
- Our study provides a comprehensive investigation of associations between all major HMO and infant growth and adiposity including several time points. Certain groups of HMOs, like the sialylated, may be associated with adiposity during the first months of lactation.
- HMO may modulate the risk of future metabolic disease. Future population studies need to address the role of specific groups of HMOs in the context of health and disease to understand the long-term impact.

INTRODUCTION

Human milk composition is individualized and dynamic with changes occurring in a single feed, throughout the day and lactation stages.[1] Many factors influence these compositional differences of human milk, including stage of lactation, maternal health, length of gestation, diet, and genotype.[2] Human milk oligosaccharides (HMOs) are the third largest solid component in human milk after lipids, and lactose[3–5] with estimated concentrations in colostrum of 20–25 g/L and mature human milk of 5–15 g/L.[5–7] HMOs are non-digestible carbohydrates consisting of a lactose molecule elongated via enzymatic linkages with one or a combination of the following monosaccharides: L-fucose, D-galactose, *N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid (sialic acid).[8] There are three main categories of HMOs in breast milk: fucosylated neutral (35–50%), sialylated acidic (12–14%), and non-fucosylated neutral HMOs (42–55%).[9,10]

Fucosyltransferase 2 (FUT2) and 3 (FUT3) enzymes are partially responsible for the different structures of oligosaccharides and are affected by genetic variations in the secretor and the Lewis blood group genes, which encode FUT2 and FUT3, respectively.[7,11–13] These genetic variations are mainly responsible for the expression or not of specific HMOs.[7,8] All FUT2-dependent HMOs contain α1,2-linked fucose, including 2′-fucosyllactose (2′-FL), lactodifucosyltetraose (LDFT), and lacto-N-fucosylpentose-1 (LNFP-I).[8] HMOs

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that are FUT3-dependent include lacto-N-fucopentaose-II (LNFP-II), lacto-N-difucosylhexose-1 (LNnDFH-I) with α1,4-linked fucose, and 3-fucosyllactose (3-FL) and LDFT with α1,3-linked fucose.[8]

Based on the presence or absence of FUT2 and FUT3 polymorphisms, four distinct milk groups with different HMO compositions have been established and described before.[14] Both FUT2 and FUT3 are active in ~70% of the population,[15] while it is rare to find both enzymes inactive.[11] The FUT2 enzyme is active in ~80% of women in Europe and America.[16] When FUT2 is active, 2′-FL constitutes ~30% of all HMOs, rendering it the most abundant.[17]

Several studies report associations between HMO composition in mother's milk and infant growth and body composition.[18–22] Notably, 2′-FL was associated with higher infant growth rates, while lacto-N-neotetraose (LNnT) was observed to be inversely related.[19–22] However, another study found substantial variations in HMOs, as seen between the high (FUT2-positive) and low (FUT2-negative) 2′-FL clusters in breastmilk do not affect the growth of breastfed infants up to 4 months.[23] In animals, feeding individually manufactured HMOs in a juvenile rat model used for safety evaluation of the manufacturing process did not reveal any significant effects on growth.[24] However, a recent in vivo study reported that 10% 2′-FL, but not 5% or lower, under high-fat diet intake induced changes in microbiota and gut metabolites linking to improvement in metabolic responses and potentially modulating hyperphagia in the animals.[25]

In order to better understand the role of HMOs in maternal milk on infant growth and body composition, this present study characterizes changes in HMO content and the subsequent effects on infants at multiple time points up to 4 months. Our study objectives were to investigate whether and how single HMOs or groups of HMOs are associated with infant anthropometry (weight, length, weight for length) or anthropometry gain, fat mass, free-fat mass, and fat mass accretion during the first 4 months of life. It is the largest, multinational study to date to investigate the longitudinal effects of HMO composition on infant growth and body composition in term-born infants. Exploratory and observational in nature, the study sought to uncover potential links in HMO composition and concentration and milk group classification to anthropometric data in infants over 4 months.

METHODS

Study design and population

The Atlas of Human Milk Nutrients study is a multicenter, longitudinal, observational cohort in which breast milk as well as multiple maternal and infant parameters were collected at six different time points post partum (V1 = 2 (0–3) days, V2 = 17 ± 3 days, V3 = 30 ± 3 days, V4 = 60 ± 5 days, V5 = 90 ± 5 days, and V6 = 120 ± 5 days). This study includes 370 healthy mother–infant dyads of any ethnicity enrolled across seven European countries, including France, Italy, Norway, Portugal, Romania, Spain, and Sweden. Institutional review and local ethical boards at all participating sites approved this study. All data were collected in 2015 and analysis is ongoing. Pregnant women were recruited and screened prior to delivery, mostly during the last trimester of pregnancy. Inclusion criteria were: (i) healthy pregnant women between ages of 18 and 40 years; (ii) pre-pregnancy body mass index (ppBMI) between 19 and 29, inclusive and defined by self-reported height and weight; (iii) intention to breastfeed at least until 4 months post partum, and (iv) agreed to the study protocol and signed informed consent form.[14] Participants were excluded if they met any of the following criteria: (i) currently participating in another trial; (ii) a medical condition preventing breastfeeding or the collection of breast milk samples; (iii) diabetes, heart problems, abnormal pregnancy conditions (e.g., hypertension), or if they were on medications related to these conditions; any feeding and eating disorders including anorexia or bulimia; or if they were unable to comply with the study procedures. We have also excluded from our analysis twins and their mothers.

Data and biological sample collection

Data for this study were collected by dedicated, trained, and certified research nurses and assistants. Maternal data were collected prior to delivery and included basic demographics, height and pre-pregnancy weight, medical history, and smoking and alcohol consumption reported elsewhere.[14] Infant data were collected at birth and included delivery mode, infant sex, gestational age, siblings, and maternal and infant anthropometry. Infant body composition and adiposity were assessed in a subset of the population by PeaPod® at selected centers.[25–27] Human milk was sampled in a standardized manner at all centers. Milk was collected at 11h00 ± 2h00 using an electric breast pump (Medela Symphony). The side of the breast selected by the mother was kept the same for the entire study and the mothers were requested to empty the breast in the previous feed or the pumping session. A single full breast was sampled at each time point, and for each, an aliquot of 10–40 mL breast milk was reserved for biochemical characterization. Colostrum, or the first time point collected, was limited to 5–10 mL. The remainder of the breast milk was kept by the mother for feeding to the infant at a later time point, if so required. Each sample was transferred to freezing tubes, labelled with a subject number and collection information, stored at −18 °C in the home freezer, transferred to the hospital (storage at −80 °C), and then shipped to Nestlé Research premises (Lausanne, Switzerland), where it was stored at −80 °C until analysis. For analysis, samples were thawed and divided into 15 individual fractions of 0.2–2 mL for the different analyses. Human milk samples were then shipped on dry ice to Neotron Spa. (Italy) for HMO analysis.

HMO quantification and milk group assignment

HMOs were quantified using liquid chromatography with fluorescence detection after labelling with 2-aminobenzamide as described in the protocol by Austin and Benet.[28] Table 1 provides a list of HMOs analyzed in this study. Using genuine HMO standards from Elicityl (France) together with the assessment of purity using quantitative nuclear magnetic resonance spectroscopy, ten HMOs were quantified: 2′-FL, 3-FL, lacto-*N*-tetraose (LNT), LNnT, 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), LNFP-I, LNFP-V, and lacto-N-neofucopentaose (LNnFP).[14] Remaining HMOs were quantified against maltotriose of known purity (Sigma-Aldrich, Germany), assuming equimolar response factors.[14]

Human milk samples can be assigned to one of four milk groups based on the presence or absence of functional fucosyltransferases, FUT2 and FUT3.[11,29] Thus, after HMOs were quantified, they were placed in one of four groups based on the concentrations of 2′-FL (combined with LNFP-I as proxy for FUT2 activity and LNFP-II as proxy for FUT3 activity). Samples in milk group 1 had high levels (>25 mg/L) of 2′-FL and (>35 mg/L) LNFP-II (secretor positive, Lewis positive), group 2 had high levels (>35 mg/L) of LNFP-II and low levels (<25 mg/L) of 2′-FL (secretor negative and Lewis positive), group 3 had high levels (>25 mg/L) of 2′-FL and low levels (<35 mg/L) of LNFP-II (secretor positive and Lewis negative), and samples in group 4 had low levels (<25 mg/L) of 2′-FL and (<35 mg/L) LNFP-II (secretor negative and Lewis negative).

Statistical analysis

Regarding HMO data, which consists of 21 HMOs, values below the level of quantification for a given parameter (HMO) were replaced by half of the corresponding limit of quantification. In order to get a single measure of the concentration of HMOs over time (from visits V1 to V6), the area under the curve (AUC) of each individual HMO was derived according to the trapezoidal method. In this respect, subjects with available HMO data for a

Table 1. Maternal and infant characteristics.

Variable	All, *N*	Secretor mothers, *N* (SD)	Non-secretor mothers, *N* (SD)	Lewis-positive mothers, *N* (SD)	Lewis-negative mothers, *N* (SD)
Number (%), *N*	357	225 (63)	58 (16)	253 (70.9)	30 (8)
Age of the mother (years)	31.3 (4.3)	31 (4.1)	32.3 (4.3)	31.3 (4.1)	31.2 (4.5)
Mode of delivery (frequency)					
Caesarean	80	58	12	62	8
Vaginal	242	167	46	191	22
Maternal ppBMI (kg/m^2)					
Mean	22.7 (2.6)	22.5 (2.4)	22.9 (2.7)	22.5 (2.4)	23.1 (2.7)
Infant gender (frequency)					
Female	150	104	20	112	12
Male	172	121	38	141	18
Parity (frequency)					
1	243	172	39	186	25
2	61	41	15	51	5
3	16	11	4	15	0
4	1	NA	NA	NA	NA
5	1	1	0	1	0
Missing	35	NA	NA	NA	NA
Gestational age (months)					
Mean	39.4 (1.2)	39.4 (1.2)	39.3 (1.2)	39.3 (1.2)	39.8 (1.2)
Infant birth weight (kg)					
V0	3.4 (0.5)	3.4 (0.5)	3.4 (0.4)	3.4 (0.5)	3.4 (0.5)
V1	3.2 (0.4)	3.2 (0.4)	3.2 (0.4)	3.2 (0.4)	3.2 (0.4)
Infant head circumference (cm)					
V0	34.5 (1.4)	34.6 (1.4)	34.5 (1.2)	34.6 (1.4)	34.7 (1.2)
V1	34.5 (1.4)	34.5 (1.5)	34.6 (1.3)	34.5 (1.4)	34.6 (1.2)
Infant birth length (cm)					
V0	50 (2.1)	50.1 (2.1)	50 (1.9)	50.1 (2.0)	49.9 (2.4)
V1	50 (2.0)	50.1 (2.0)	50 (1.8)	50.1 (1.9)	49.8 (2.4)
Fat mass (kg)					
V1	0.3 (0.2)	0.3 (0.1)	0.3 (0.2)	0.3 (0.1)	0.4 (0.1)
Fat mass (%)					
V1	10.2 (3.6)	10.3 (3.6)	10.2 (3.7)	10.2 (3.6)	10.8 (2.8)

given HMO at both V1 and V6 time points were considered for AUC computation.

Anthropometric data consisted of infant weight, length, head circumference, and BMI (derived from infant weight and length) at all time points. In addition, weight for age, weight for length, length for age, head circumference for age, and BMI for age at each visit were computed with respect to the World Health Organization (WHO) reference data.[30] Anthropometrics gain (weight gain, length gain, and head-circumference gain) between V1 and V6 were computed by taking the difference of the growth indicator value between V6 and V1 and dividing it by the number of days between V1 and V6. Similar computation was done for the gain in *z*-scores between V1 and V6.

For infant body composition, measures of infant fat mass and fat-free mass were taken at each visit. The fat mass index (FMI), fat-free mass index, and the fat mass accretion from V1 to V6 were derived from fat mass. Fat mass accretion was defined as the difference of fat mass between V6 and V1 divided by fat mass at V1.

To assess the association between HMOs and the infant anthropometry and body composition parameters, Spearman's rank-order correlations between the AUC of individual HMOs and each parameter of anthropometry and body composition measured at V6 (4 months) was computed. Moreover, Spearman's correlations between individual HMO concentration at a specific time point and growth parameters at the same time point were also performed (results not shown).

Furthermore, the correlation structure between HMOs was investigated by means of a principal component analysis (PCA). PCA was performed on AUCs of individual HMOs. Those AUCs were mean centered and scaled (i.e., divided by their standard deviation) before PCA computation. Results of PCA can be used to get an overview of the association between HMOs and growth parameters by projecting subjects (pairs of mother and children) and the variables (HMO AUCs) on a two-dimensional space spanned by two principal components (the first two principal components presented here) and by color-coding subjects according to their anthropometric and body composition data (we divided the population into groups—low, normal, and high—according to tertiles of a given growth indicator).

The association between AUCs of individual HMOs and fat mass accretion (from V1 to V6) was assessed by linear regression. More specifically, the fat mass accretion was separately regressed on the AUC of each individual HMO. The model included fat mass at V1, pre-pregnancy BMI, sex, and infant birth weight as additional covariates.

Finally, the distribution of each HMO AUC was compared between infants, stratified by sex or not, who fell within the upper and lower 25th percentiles of the weight-for-length *z*-score gain. To this end, a (two-sided) Wilcoxon rank-sum test was used.

The statistical significance level for tests was considered at 0.05. Given the exploratory nature of the study, no adjustment for multiplicity (in the context of hypothesis testing) was performed. Statistical analyses were conducted using the R statistical software version 3.4.1.

RESULTS

In total, 370 pregnant women (mean age = 31.2 years, SD: 4.1) were screened for enrollment in this European study. The analysis was performed on a subset of 357 mothers (and their infants), after removal of non-eligible mothers (with respect to the inclusion/exclusion criteria) and the ones who delivered twins (see Fig. 1). Maternal demographics were previously described.[14] Pre-pregnancy height and weight were self-reported and used to calculate the ppBMI. Mean pre-pregnancy maternal weight was 61.7 kg (SD: 7.7) and, based on calculated BMI, 79% of participants were normal weight and 21% were considered overweight according to the WHO criteria.[31] There was no difference in ppBMI between the milk groups and other maternal characteristics were similar, with the exception of maternal height ($P = 0.022$) and distribution by country ($P = 0.005$) as described previously.[14] Seventy-four percent of mothers gave birth for the first time. Milk group distribution varied by country and was as follows: 72% of samples were assigned to milk group 1 (FUT2+, FUT3+), 17.6% to milk group 2 (FUT2−, FUT3+), 6.9% to milk group 3 (FUT2+, FUT3−), and 3.5% to milk group 4 (FUT2−, FUT3−) (Table 1).

Anthropometric data were available for 322 infants at birth and 224 infants at 4 months (Fig. 1). The main reasons for dropout included the following: 37 women gave no explanation, 50 women reported insufficient milk supply, 57 women stated other reasons, including feeding infant formula for at least 7 consecutive days, choosing to withdraw from the study, and cessation of breastfeeding to return to work.[14] Maternal and infant characteristics are provided in Table 2. Mean gestational age was 39.4 weeks (SD: 1.2) and 53.4% were male. Over 25% of infants were delivered via Cesarean section (C-section).

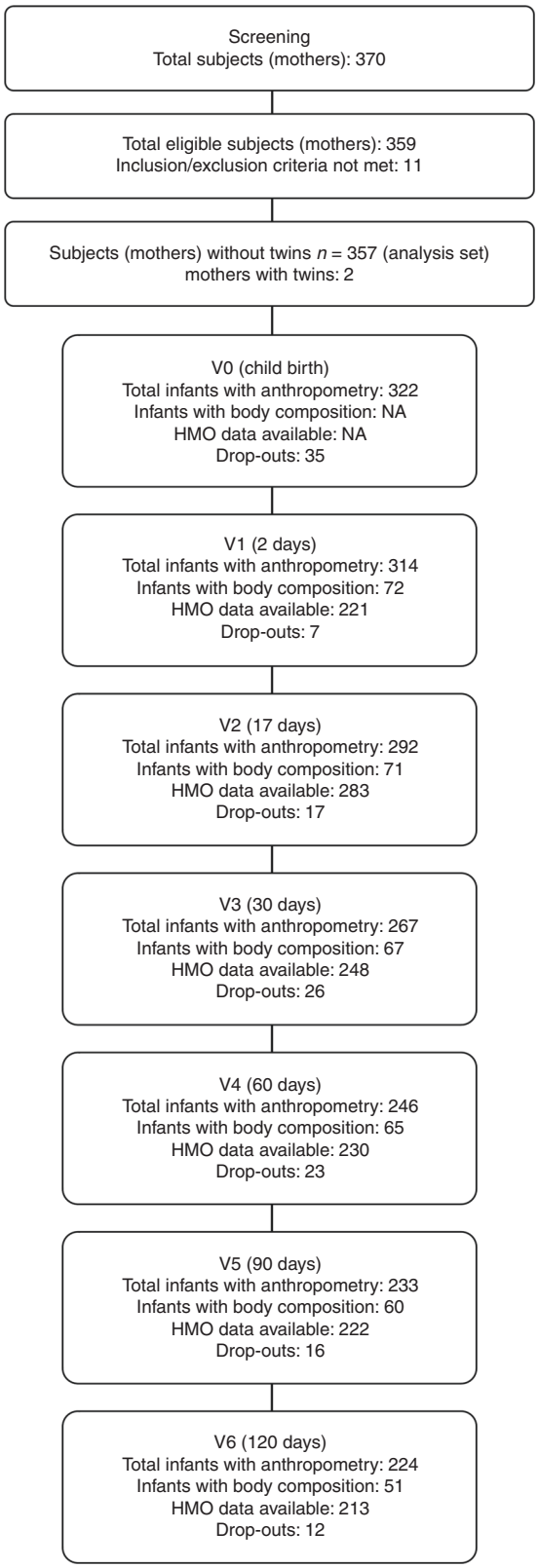

Fig. 1 Schematic of study design and flow. After screeening and applying inclusion and exclusion criteria, removing mothers with twins, $N = 357$ mothers remained, whi were followed from birth until 120 days postpartum.

Table 2. The human milk oligosaccharides analyzed in this study.

Name	Abbreviation
2′-Fucosyllactose	2′-FL
3-Fucosyllactose	3-FL
6′-Galactosyllactose	6′-GL
3′-Sialyllactose	3′-SL
6′-Sialyllactose	6′-SL
Lactodifucosyltetraose	LDFT (DiFL)
Lacto-N-tetraose	LNT
Lacto-N-neotetraose	LNnT
A-tetrasaccharide	A-Tetra
Lacto-N-fucosylpentaose-I	LNFP-I
Lacto-N-fucosylpentaose-II	LNFP-II
Lacto-N-fucosylpentaose-III	LNFP-III
Lacto-N-fucosylpentaose-V	LNFP-V
Lacto-N-neofucosylpentaose-V	LNnFP-V
Sialyllacto-N-tetraose b	LSTb
Sialyllacto-N-tetraose c	LSTc
Lacto-N-difucosylhexaose-I	LNDFH-I
Lacto-N-neodifucosylhexaose	LNnDFH
Disialyllacto-N-tetraose	DSLNT
Monofucosyllacto-N-hexaose-III	MFLNH-III
Difucosyllacto-N-hexaose a	DFLNHa

Infant anthropometric data and HMO (Table 2) composition was assessed across six time points: V1 = 2 (0–3) days, V2 = 17 ± 3 days, V3 = 30 ± 3 days, V4 = 60 ± 5 days, V5 = 90 ± 5 days, and V6 = 120 ± 5 days.[14] All infants grew within the normal range according to WHO growth charts. These charts are displayed as Figs. 2 and 3 and differentiate secretors versus non-secretors. Figure 4 shows the change in FMI over all six time points by secretor status. Supplementary Figs. S1–5 compare growth of those that were identified as Lewis positive or negative.

A Spearman's rank-order correlation was run to determine the relationship between HMO AUC over the first 4 months of lactation and anthropometric measures at 4 months of age (V6) (Supplementary Figs. S6A, B). The change in individual HMO concentration over time had little effect on anthropometric data at 4 months. 3′-SL AUC was negatively correlated with length ($rs = -0.15$, $P = 0.049$), and while there were no correlations with length or weight for length, both MFLNH-III and LNFP-III AUCs were positively correlated with head circumference ($rs = 0.18$, $P = 0.018$ and $rs = 0.16$, $P = 0.041$, respectively). The change in HMO concentration over time also had little effect on anthropometric data during that period. A-Tetra AUC had a negative correlation with the change in head circumference ($rs = -0.16$, $P = 0.039$). LNnT AUC had a negative correlation with the change in length ($rs = -0.18$, $P = 0.02$). LSTc AUC had a positive correlation with weight for length ($rs = 0.19$, $P = 0.015$).

Fat mass accretion and the FMI were assessed as clinically relevant indicators for body composition in young infants and available for a subset of infants. Fat mass accretion was measured at each of the six clinic visits from birth to 4 months in 42 infants and the FMI was calculated at the study end point at 4 months of infant age (V6) in 44 infants. To explore possible relationships between fat mass accretion or FMI with the AUC of individual HMOs, we performed a PCA biplot of the AUC of individual HMOs over all six visits and coded infants in tertiles of low, medium, and high for both

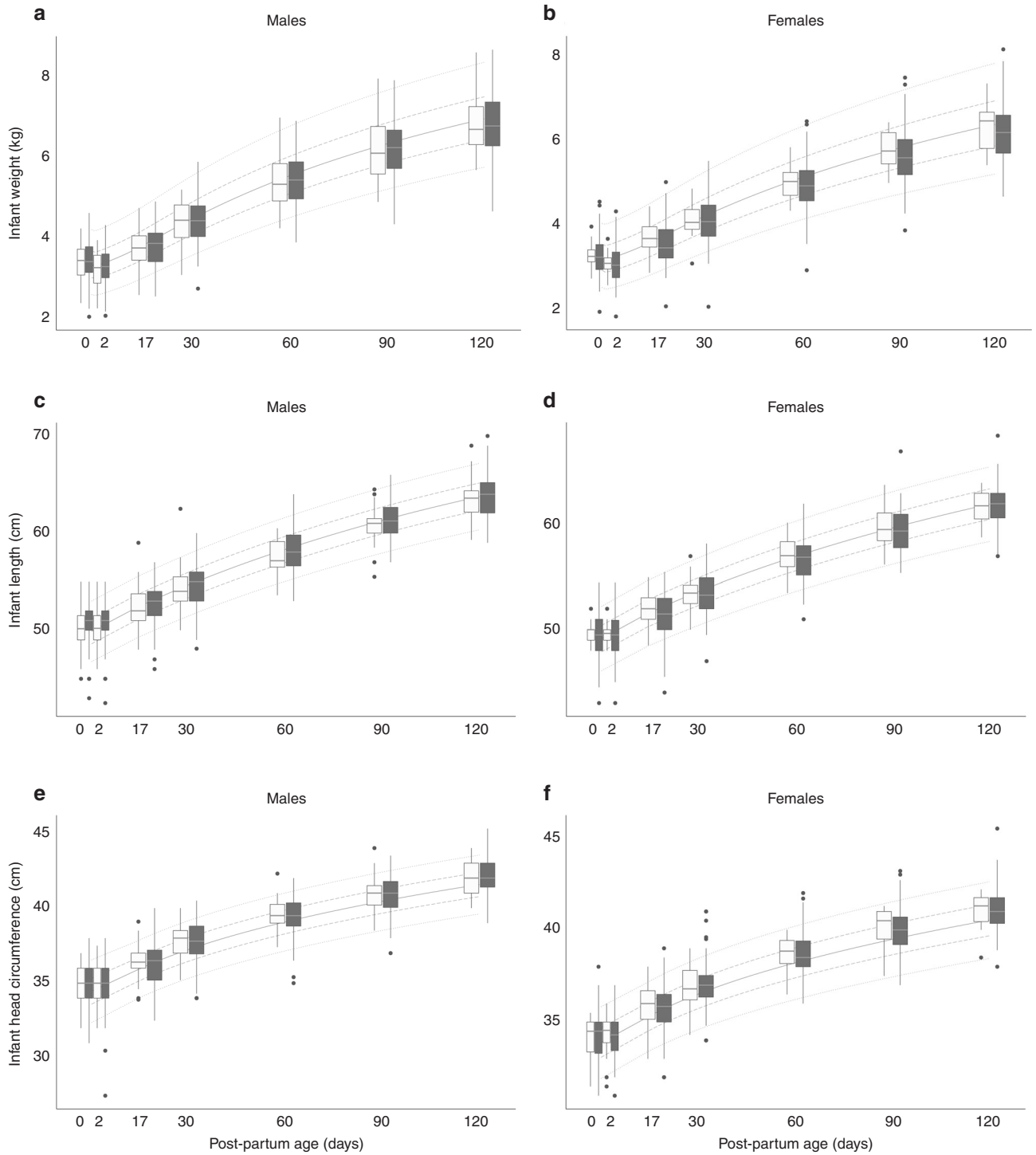

Fig. 2 Infant anthropometry from birth to 120 days after birth. a, **b** Infant weight (kg) of male (**a**) and female (**b**) infants plotted on WHO growth charts by secretor status. SEneg secretor negative (white box plot), SEpos secretor positive (back box plot). WHO percentiles are depicted (5th, 25th, 50th, 75th, 95th). **c**, **d** Infant length (cm) of male (**c**) and female (**d**) infants plotted on WHO growth charts by secretor status for all six time points. SEneg secretor negative (white box plot), SEpos secretor positive (back box plot). WHO percentiles are depicted (5th, 25th, 50th, 75th, 95th). **e**, **f** Infant head circumference (cm) of male (**e**) and female (**f**) infants plotted on WHO growth charts by secretor status for all six time points. SEneg secretor negative (white box plot), SEpos secretor positive (back box plot). WHO percentiles (5th, 25th, 50th, 75th, 95th) are depicted.

fat mass accretion (Fig. 5a) and FMI (Fig. 5b). The first two components of the PCA explained 49 and 48.8% of the variability. In both PCA biplots, the population means are close to the center, indicating that neither fat mass accretion nor FMI is related to a specific HMO intake illustrated by the AUC of each individual HMO. The quality of the representation of the AUC for each individual HMO for the principal components 1 and 2 are depicted by the arrows on the PCA biplot. Similarly, an observation of each individual infant in each of the tertiles for fat mass accretion or FMI shows a very large overlap and no separation. Collectively, the PCA analysis

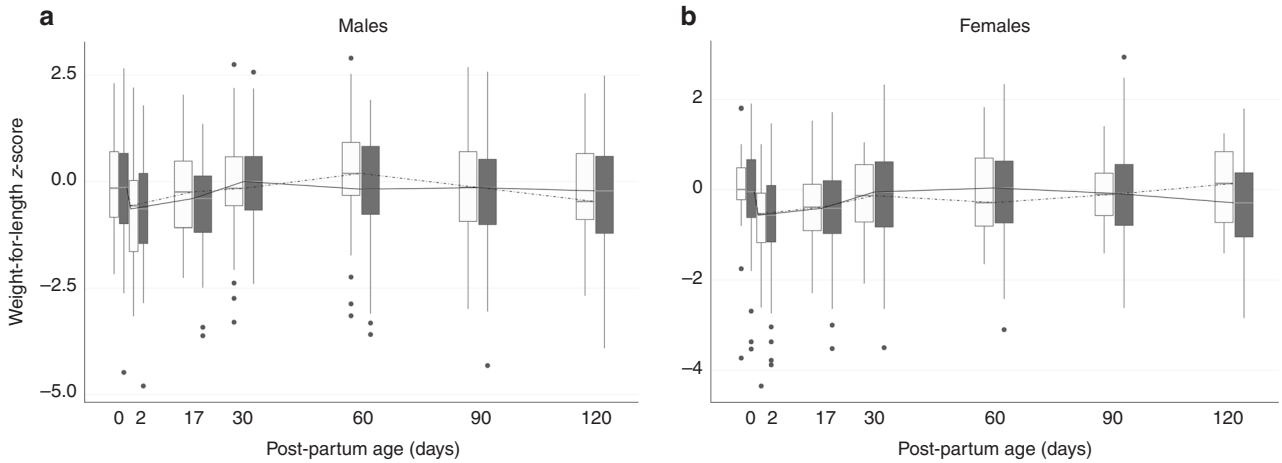

Fig. 3 Weight-for-length from birth until 120 days after birth. Infant weight for length *z*-scores of male (**a**) and female (**b**) infants by secretor status for all six time points. SEneg secretor negative (white box plot), SEpos secretor positive (back box plot).

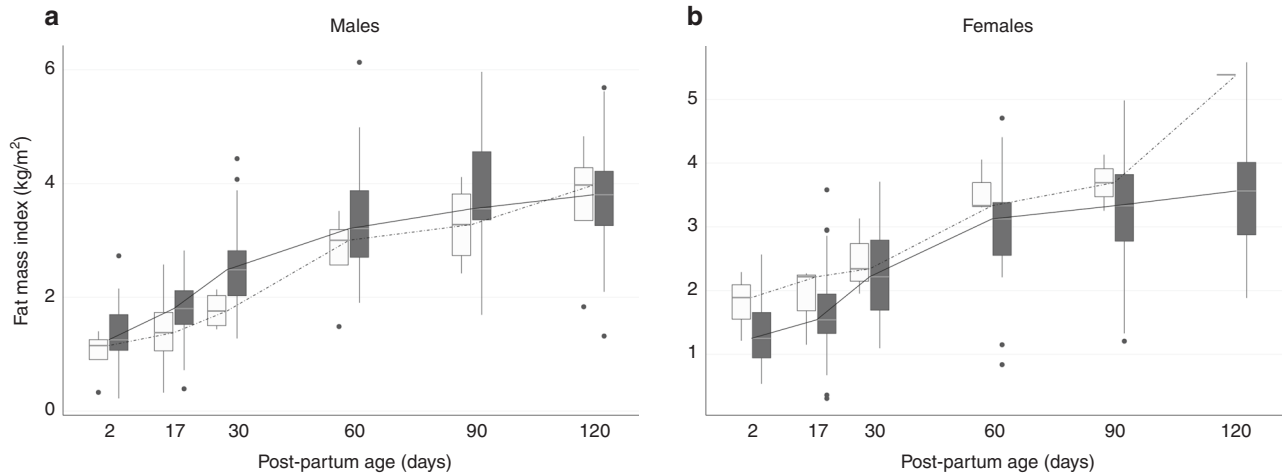

Fig. 4 Fat mass composition from birth until 120 days after birth. Infant fat mass index (kg/m^2) of male (**a**) and female (**b**) infants for all six time points by secretor status for all six time points. SEneg secretor negative (white box plot), SEpos secretor positive (back box plot).

did not reveal any obvious relation between fat mass or FMI with the AUC of any single HMO.

By regressing fat mass accretion on individual HMO AUC and other potential confounding variables (ppBMI, sex, infant birth weight, and fat mass at V1), two HMOs were found to potentially have an effect. MFLNH-III AUC appears to be predictive with an estimated increase of 0.0000956 in fat mass accretion due to an increase of one unit of MFLNH-III AUC ($n = 42$, $r^2 = 0.590$, adjusted $r^2 = 0.534$, SE ≤ 0.001, $P = 0.017$). Further, LNnDFH AUC was potentially influencing fat mass accretion with an estimated increase of 0.00044 in fat mass accretion due to an increase of one unit of LNnDFH AUC ($n = 42$, $r^2 = 0.572$, adjusted $r^2 = 0.513$, SE ≤ 0.001, $P = 0.042$).

We explored the distribution of each HMO AUC by comparing it between infants who fell within the upper and lower 25th percentiles of the weight-for-length *z*-score gain (Fig. 6). These plots are made to compare the two groups of subjects (highest and lowest quartiles) side by side with respect to each HMO. In Fig. 6, results of only those HMOs associated with differences in growth rate for males and/or females are shown. When males and females were combined, 3′-SL AUC and LSTc AUC had significant different distributions (in terms of location) between infants in the highest and lowest quartiles of the weight-for-length *z*-score gain ($P = 0.017$ and $P = 0.006$, respectively). Infants in the upper 25th percentile (25 males and 17 females) had higher 3′-SL and LSTc concentrations. This group included 31 secretor-positive and 11 secretor-negative infants. Infants in the lower 25th percentile included 25 males and 17 females and 33 secretor-positive and 9 secretor-negative infants. Similar findings were observed using the same analysis in males with both 3′-SL and LSTc potentially affecting growth rates ($n = 25$, $P = 0.003$, and $P = 0.003$, respectively). However, these results were not seen in females, for which we observe a difference in LNnFP between the two groups ($n = 17$, $P = 0.045$) (Fig. 6).

DISCUSSION

The period between conception through the first 2 years of life, the first 1000 days, is critical for human growth and development.[32] Infant growth can be affected by a number of environmental, genetic, epigenetic, metabolic, and microbial factors.[33] Undernourished children display an abnormal gut microbiota, which has led some to conclude that a disrupted microbiota may impair normal postnatal growth and affect nutritional status.[34,35] Breast milk provides the optimal nutrition for the growing infant and is rich in bioactive substances contributing to a beneficial gut microbiota including HMOs.[14,29,36–38] The present study adds to our understanding of possible physiological meanings of breastmilk HMO compositional variations for breastfed, term-born, and generally healthy growing

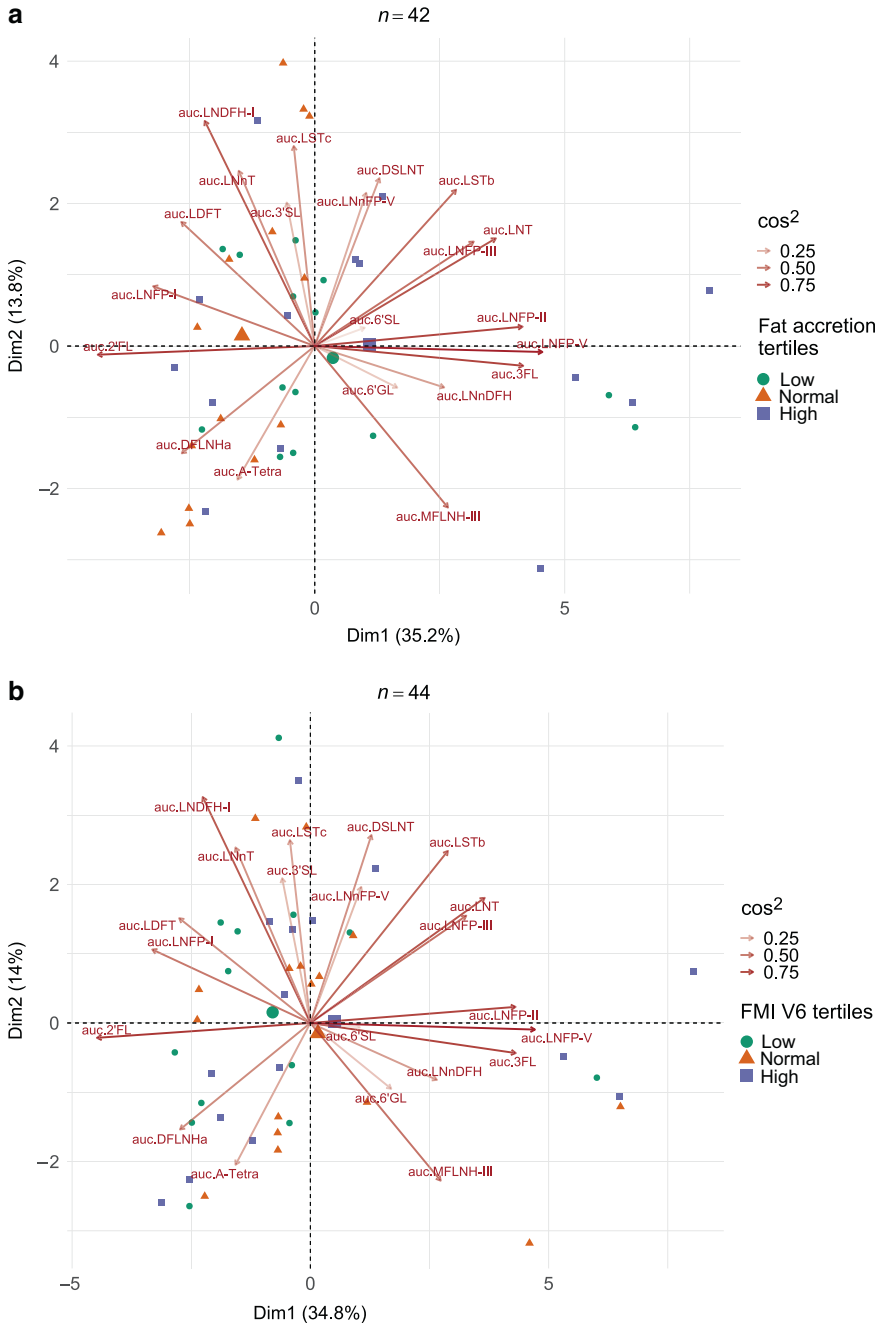

Fig. 5 HMOs area under the curve (AUC) calculated for the entire lactation period and fat mass index at 120 days after birth. Biplots of principal components 1 and 2 (dimensions (Dim)1 and Dim2) where individuals and additionally the variables (AUC of each individual HMO) are projected on the plane of the individuals colored for fat mass accretion between V1 and V6 with $n = 42$ infants (**a**) and fat mass index (FMI) at V6 with $n = 44$ infants (**b**). Individual infants are shown in the analysis with those in the lower tertile appearing as small, green circles, those in the middle tertile as small, orange triangles, and those in the high tertile as small, blue squares. The larger version of each icon represents the population mean. Arrow lengths and color intensity are indicated by the $\cos^2$ with 1 being highest, which depict the quality of representation of the AUC of each individual HMO for PC 1 and 2.

infants. Globally, HMOs seem to have neutral or only moderate effects on early infant growth parameters, including fat mass accretion that is related to the future development of overweight or obesity. Noteworthy, sialylated HMOs, like 3′-SL and LST, showed significant associations in our study to weight for length, in line with previous studies including malnourished infants.[20,21] These findings provide directions to future studies to investigate the role of specific sialylated HMOs in catch-up growth for preterm and malnourished infants.

HMO composition is affected by several factors with maternal genotype-like secretor and Lewis status and time of lactation being the most influential.[14,39,40] Generally, HMO composition is relatively similar between preterm and term milk.[7,41–44] However, some significant differences in HMO concentration were noted between preterm and term milk at equivalent post-partum age, including higher 3′-SL in preterm milk across the assessed 2 months of lactation. To what extent such compositional differences may indicate a milk adaptation to specific infant

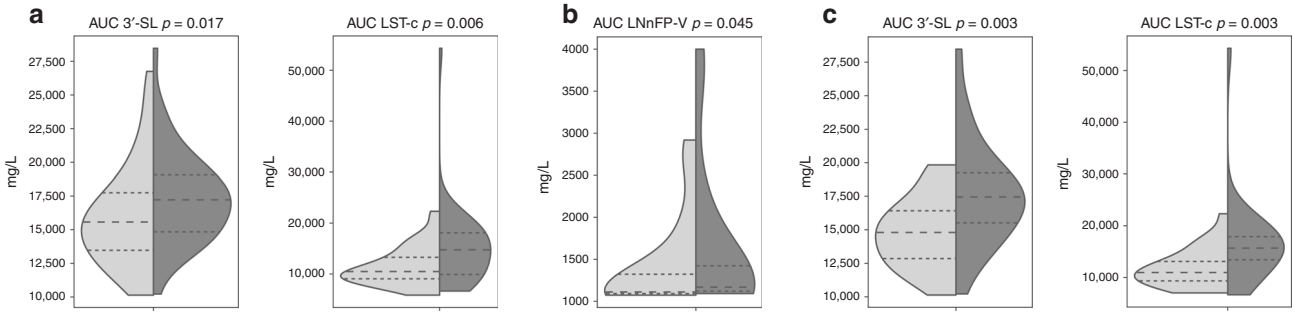

Fig. 6 Growth rate association with HMOs. Weight for length gain rate by highest and lowest quartile by HMO AUC over the first 4 months of lactation for all (**a**), female (**b**), and male (**c**) for those HMOs with statistically significant differences.

requirements or reflect the maternal physiological status is not known.

HMOs play an important role in the development of the infant's gut microbiome.[45–48] This may affect nutrient efficiency and gut health and consequently infant growth. Some variability in the gut ecology exists among breastfed infants due to the ability of specific bifidobacterial strains to use fucosylated HMOs or not, which affects stool acidification via short-chain fatty acid production.[49] Such changes may impact mineral absorption for example.[50] In addition, disrupted gut microbiota due to the exposure to antibiotics during the early life, especially the first 6 months, represents a risk for later obesity.[51] Hence, the gut microbiota should be considered in future studies as an additional element when investigating HMO composition and infant growth and body composition outcomes.

Observations on HMOs and growth

Breastfeeding may reduce the risk of overweight and obesity, but not all evidence is equally strong.[52] Some exclusively breastfed infants experience excessive weight gain during the first 6 months and may later have a catch-down effect after starting solid foods.[53–55] Thus, in an attempt to understand what variable components of human milk might result in weight gain, recent studies investigated the link between HMO composition and the effects on infant growth and body composition.[18–21]

Small studies link HMOs to growth in early infancy affecting both weight-for-age *z*-scores (WAZ) and length-for-age *z*- scores (LAZ) depending upon the specific HMO.[18,20,21] Similar to our finding, two studies observed a positive association between sialylated HMOs and growth.[20,21] In one of them, a higher proportion of 3′-SL in breast milk corresponded to higher WAZ in infants ($n = 33$), while, contrary to what we found, a higher proportion of LSTc contributed negatively to WAZ.[20] In this latter study, the fucosylated HMO LNFP-I was also positively associated with LAZ. Because LNFP-I concentration was greater in the milk of mothers whose infants had less sick days, the authors suggested that LNFP-I may be involved in sparing resources that would be used to fight off infection to help maintain growth. In contrast, another small study of 25 mother/infant dyads in the United States reported a higher concentration of LNFP-I to be associated with lower infant weight, lean mass, and fat mass.[18] On the other hand, in the same study, DSLNT, LNFP-II, and fucosyl-disialyl-lacto-*N*-hexaose were associated with a greater body of fat mass and higher LNnT concentrations were associated with lower body fat.[18] Apart from the link between 3′-SL and growth, our study did not confirm any of the previously reported associations.

An exploratory study of 13 high weight-gain (HW) and 17 normal weight-gain (NW) breastfed infants found that HMO composition in breast milk of HW infants was significantly different to that of NW infants.[19] At 5 months, total HMO concentration and HMO-bound fucose were positively associated with FMI and weight velocity from 0 to 5 months. 2′-FL, the most abundant secretor-dependent fucosylated HMO in human milk, was also positively associated with FMI and weight velocity.

Somewhat similar findings were reported in another longitudinal cohort study that examined the relationship between HMOs at 3 months of life and child growth through 5 years, along with the mothers ppBMI in 802 mother–child dyads from Finland.[56] Interestingly, in a ppBMI-adjusted model, LNnT and LSTb were negatively correlated with both length and weight *z*-scores from 3 months through 5 years and 3 to 12 months, respectively. Conversely, 2′-FL was positively correlated with length *z*-scores through 5 years and with weight *z*-scores between 3 to 12 months. Additional positive correlations to weight *z*-scores were reported for concentrations of 3-FL and 3′-SL through 5 years and LDFT from 3 to 12 months. The results on infant growth showed that by 3 months of age, the highest and lowest quartile of 2′-FL, LNnT, and their ratios are already associated with different growth trajectories. Our study that examined associations through 4 months of age is a relevant complement to this study. However, our study did not reveal any association between 2′-FL or LNnT and infant anthropometry. While we observed some correlations between anthropometrics and HMOs, associations were generally weak and only a few, like with 3′-SL, were also observed in other studies. The lack of a relationship between secretor-positive (e.g., 2′-FL, LNFP-I) and secretor-negative HMOs and growth during the first 4 months of age in the present study was similar to a previous assessment in an observational study of 50 mother–infant pairs.[23]

The impact of individual HMOs on growth may be more easily assessed if they are considered in the absence of other varying factors. Randomized controlled trials with infant formula provide such an opportunity. Today, several such trials with 2′-FL and LNnT (at 1 and 0.5 g/L) or 2′-FL (at 1 or 0.2 g/L) noted age-appropriate growth comparable to non-supplemented formulas and also to breastfed infants.[17,57–60]

We compared the HMO AUC concentrations for infants who experienced the least and greatest growth gains (lower and upper 25th percentiles) based on weight for length. Small differences were observed in the 3′-SL AUC and LSTc AUC. Interestingly, in animal models of infant undernutrition, sialylated oligosaccharides were found to promote growth via modulation of the gut microbiota.[21] These observations together with our data and previous clinical observations point to a specific role of individual HMO to restore and ensure adequate infant growth. Our study included only well-nourished and healthy growing infants. It would be interesting to test our observations in cohorts or interventional studies of infants falling outside the optimal growth trajectories to confirm our hypothesis.

Limitations

This observational, longitudinal cohort study has several limitations including the nature of the design that restricts the findings to associations without establishing causation. The main limitation of this study is the 4-month duration. However, after 4 months it becomes increasingly difficult to control for the introduction of complementary foods and a mixed formula and breastmilk diet. Although breastmilk intake was not quantified, a similar intake is assumed since participants were excluded if they followed a mixed diet of formula and breastmilk for longer than 7 days and complementary foods had yet to be introduced. Other limitations include a low number of infants with body composition measures, since only selected sites collected these data and lack of pediatric sickness data that could affect the growth outcomes. The ppBMI of mothers fell within the normal BMI range, which we know is not necessarily representative of the population. Additional studies on mothers who are outside this range could help determine how ppBMI could serve as a confounding variable on HMO AUC and infant anthropometric data.

Strengths

The strengths of the study include the large sample size for anthropometry and the multicenter design. The longitudinal nature of the study with standardized sampling of breastmilk at multiple time points and adjustments for potential confounding variables allows for a more reliable interpretation of the associations.

CONCLUSION

Individual HMO AUC during the first 4 months appears to have no or only moderate effect on infant growth and body composition during this time of exclusive breastfeeding in term-born, healthy growing infants. Correlations to anthropometric data and changes in growth velocity were weak, while a regression analysis of HMO AUC and fat mass accretion revealed a moderate effect. Also, maternal secretor status and respective HMO variations do not appear to play a significant role in infant growth during the first 4 months of age. Additional studies evaluating a broader range of maternal ppBMI, as well as controlled feeding studies extending beyond 4 months and coupled with further metabolic and gut microbiota analysis, are important follow-ups to the present study. Notably, selected sialylated HMOs may be interesting targets in situations of growth failure and where catch-up growth is important.

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AUTHOR CONTRIBUTIONS

A.B., L.L., C.C., S.A., E.C.-G., M.A., I.A.-J., A.B.P., M.J.C., M.G.S., G.M., C.M.-C., T.S., S.-M.S., M.V., T.R., C.B., J.-C.P., and M.D., had substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. A.B., N.S., R.A., M.V., M.A., M.J.C., C.M.C., and M.D. drafted the article and/or revised it critically for important intellectual content; and all authors had a final approval of the version to be published.

ADDITIONAL INFORMATION

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Statement of consent: The participants provided a written informed consent form to participate in the study after receiving explanations and having read and understood the purpose and the objectives of the study in their respective local languages. The study was registered at www.clinicaltrials.gov with the identifier NCT01894893.

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