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Breast milk composition and infant metabolism

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Breast milk composition and infant metabolism

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Editorial: Breast milk composition and infant metabolism

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Editorial on the Research Topic
Breast milk composition and infant metabolism

Nutrition in early life is of fundamental importance in an infant's future health. Breast milk is the first natural functional food for infants and the "gold standard" for infant feeding (1). Breast milk not only contains various nutrients suitable for infants' digestion and absorption, but also contains a variety of bioactive substances, such as immunoglobulins, hormones, oligosaccharides, and bacterial constituents (2). These functional components in breastmilk are known to exert a series of beneficial effects, including reduced risk from infections and promoting various aspects of postnatal development. Although the mechanisms underlying some of these benefits have been elucidated, the origins of others that have been reported, such as influence on neurological, immunological, and metabolic outcomes remain more obscure.

Breastmilk does not stand alone. Breast milk is produced by the mammary glands of future mothers and lactating mothers. The effect of the mother's diet, nutrition and health status, and environmental exposures on the composition of breast milk, and infant developmental outcome, are also issues that need to be further understood. Additionally, as a dynamic functional food for infants, human milk might adapt to meet infants' needs under preterm, infectious, and others.

In this Research Topic, we would like to present reviews and original articles covering the latest developments in studying breast milk composition, formula milk, and infant metabolism. Studies discussing the health issues mentioned above are suitable for consideration in this Research Topic. We hope that our Research Topic will contribute to deepening our knowledge in this area and provide the basis for creating new prophylactic and therapeutic standards.

In this special e-collection there are 13 papers covering the above mentioned aspects.

Breast milk contains a variety of bioactive components, and the composition of breast milk has always been the focus of attention in maternal and infant research. Ten papers out of 13 (76.9%) were related to this topic. Poulsen et al. performed metabolomic analysis of mature breast milk from mothers who delivered at term after 1 month and found that the breast milk metabolome was dynamic during maturation, which compensated for the deficiencies of mature milk studies. Lactopontin is a protein present in breast milk and related to neonatal immunity. The study by Zhu et al. described in detail the dynamic changes of Lactopontin in breast milk of newborn mothers at different gestational ages. Vitamin E is another component of breast milk that is related to infant immunity, but the content of vitamin E in breast milk during different lactation periods has not been systematically described. To clarify this issue, Xi et al. in their meta-analysis summarized the currently published articles in support of maternal and infant protective strategies regarding

vitamin E. Breast milk sialic acid is very important for the development of the nervous system of infants. Previous studies generally believed that diet may have an impact on the content of sialic acid in breast milk. Xie, Xu et al. found no correlation between the content of sialic acid in breast milk and diet in their study of 33 mothers in Xiamen, China. Iodine is very important in neonatal neurodevelopment, and breast milk is the main way for newborns to obtain iodine. Guo et al. comprehensively evaluated the characteristics and predictors of breast milk iodine concentration (BMIC) and proposed the best predictor of BMIC. Breast milk exosomes have always been a hot topic in the study of breast milk composition. Cho et al. conducted a comprehensive analysis of breast milk exosomal miRNAs from obese mothers and pointed out the differences between them and those from normal weight mothers, which pointed out a new direction for the study of the effect of maternal obesity on infants.

The impact of the large number of bioactive substances in breast milk on the infant, including body composition, hormonal and cytokine profiles, and microorganisms and metabolites in feces, has been the focus of attention. Previous studies have shown that human milk oligosaccharides play an important role in the formation of intestinal microecology in infants, but few studies have identified the specific oligosaccharides. Data from the study of the Cambridge Infant cohort by Chichlowski et al. showed that higher concentrations of 2'fl and LNFP1 could promote the proliferation of bifidobacteria and could increase the abundance of intestinal flora in infants. Preterm birth is the leading cause of death in children under 5 years of age (3). Studies on the association between various breast milk components and the growth and development of preterm infants have always been a key part of maternal and infant research. Glucocorticoids play an important role in the regulation of hypothalamic-pituitary-adrenal (HPA) axis in infants, especially in premature infants. Previous studies have only focused on the role of breast milk Glucocorticoids (GCs) in very early preterm infants. The study by Muelbert et al. focused on the role of breast milk GCs in middle and late preterm infants, which was a supplement to the effect of breast milk GCs on preterm infants.

Whether there are cross-generational effects of lactation diet has been inconclusive. Vargas et al.'s study on mice showed that protein restriction during lactation led to cross-generation effects of metabolic dysfunction in mice, which provided new research ideas for subsequent studies. Bronchopulmonary dysplasia (BPD) is a serious chronic lung disease that affects the long-term health of newborns. The main pathogenic mechanism is oxidative stress (OS). The review by Yang et al. summarizes the variety of antioxidant factors contained in breast milk and suggests a new direction for the treatment of BPD.

Due to various reasons, many newborns partially or completely rely on various formulas (4). How to make formula more similar to breast milk has been one of the research hotspots in this field. At present, the underlying mechanisms of osteopontin (OPN), 2′-fucosyllactose (2′-FL) and docosahexaenoic acid (DHA) on neural cell development are still unknown. Xie, Zhang et al. showed that OPN, 2′-FL and DHA have the effect of promoting myelination of nerve cells, which can provide a theoretical basis for further optimization of infant formula. Fatty acid (FA), another major component of breast milk, may be affected by a variety of factors. Ni et al.'s study found that a variety of socio-demographic factors (e.g., maternal age, gestational weight, etc.) were related to total FA, while sn-2 FA content was almost not affected, which may provide ideas for the development of infant formula.

In summary, the results of the above studies and reviews represent a large amount of relevant data on the dynamic changes of human milk (HM) composition and its metabolism in infants. Despite all the available literature and evidence relating to this extremely important topic, the papers published in this e-book clearly demonstrate that there are still many aspects that need to be clarified and understood in relation to HM components and infant metabolism. After reading this book, some topics, such as the dynamics of HM composition, the effects of HM composition on infant metabolism, short-and-long term effects of breast milk composition on infant diseases, formula development based on breast milk composition, will become clearer to the reader and reinforce the belief that HM represents the best food for feeding all newborns, including preterm infants.

Author contributions

DM wrote the introduction and the conclusion. BC wrote the central part with comments to the cited papers and references. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Dynamic Changes in the Human Milk Metabolome Over 25 Weeks of Lactation

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Human milk (HM) provides essential nutrition for ensuring optimal infant growth and development postpartum. Metabolomics offers insight into the dynamic composition of HM. Studies have reported the impact of lactation stage, maternal genotype, and gestational age on HM metabolome. However, the majority of the studies have considered changes within the first month of lactation or sampled with large intervals. This leaves a gap in the knowledge of progressing variation in HM composition beyond the first month of lactation. The objective of this study was to investigate whether the HM metabolome from mothers with term deliveries varies beyond 1 month of lactation, during the period in which HM is considered fully mature. Human milk samples (n = 101) from 59 mothers were collected at weeks 1–2, 3–5, 7–9, and 20–25 postpartum and analyzed using ^{1}H nuclear magnetic resonance spectroscopy. Several metabolites varied over lactation and exhibited dynamic changes between multiple time points. Higher levels of HM oligosaccharides, cis-aconitate, O-phosphocholine, O-acetylcarnitine, gluconate, and citric acid were observed in early lactation, whereas later in lactation, levels of lactose, 3-fucosyllactose, glutamine, glutamate, and short- and medium-chain fatty acids were increased. Notably, we demonstrate that the HM metabolome is dynamic during the period of maturity.

Keywords: human milk, metabolomics, term delivery, lactation, NMR spectroscopy

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INTRODUCTION

Feeding a full-term infant with mother's milk provides macronutrients and non-nutritional compounds accommodating the infant's needs in the early neonatal period following birth, including supporting growth, immunological development, and gut maturation (1, 2). Human milk (HM) contains immunological compounds interacting with the developing infant's immune system (3), non-nutritional compounds involved in immunological and growth-related metabolic mechanisms (2, 4), and vitamins and minerals (5), as well as prebiotic glycans, such as human milk oligosaccharides (HMOs), providing energy for the development of the gut microbiota and ensures antimicrobial activity in the gut (6, 7). Thus, the composition of HM is complex and is in addition dynamic over time, with intraindividual variation observed for various types of constituents (8–12).

Human milk metabolomics is still a developing scientific field with continual changes and improvements in analytical technology. One of the most used techniques is NMR spectroscopy as this method is robust, quantifies the metabolites in high concentration, and yields a good

coverage of milk metabolites across different metabolite classes (13). Recently, metabolomic studies have contributed to insight into low-molecular weight compounds of HM and their variation (8, 14). Studies have particularly reported variations in mono- and disaccharides, HMOs, free amino acids, free fatty acids, lipids, and intermediates of energy metabolism in HM (15–17), and some of these constituents have implications on infant development. For instance, HMOs are the prebiotic components important for immune system development and gut microbial colonization (6), glutamine and glutamate act as neurotransmitters for the brain besides from serving as nitrogen sources (18), and choline is important in brain development and phospholipid synthesis among others (19).

Studies on HM metabolome provide an opportunity for understanding the impact of maternal genotype, disease, and lifestyle on HM composition reflected by the metabolome (8, 14). The composition of metabolites in HM varies over lactation, with increasing levels of lactose and short- and medium-chain fatty acids and decreasing levels of HMOs when progressing from colostrum (first day after birth) to mature milk (recognized as 4 to 6 weeks postpartum) (2, 15). Apart from the variation during the lactation period, metabolomic studies have demonstrated variation in HM metabolome dependent on maternal genotype, impacting the chemical composition of HMOs in the milk (20). More than 150 different structures dependent on glycosidic linkages and the composition of carbohydrate monomers have been characterized (21). In particular, secretor status and Lewis gene type of the mother influence the chemical structures of HMOs available in HM (12). Mothers classified as secretors excrete HMOs with α1-2 fucosylation, of which 2-fucosyllactose (FL) and lacto-N-difucohexaose (LNDFH) I are among the quantitatively most dominant, whereas the milk of non-secretors lacks these structures. In contrast, non-secretor mothers secrete milk dominated by 3-FL, lacto-N-fucopentaose (LNFP) II and lacto-N-tetraose (LNT) (22). Finally, Lewis negative mothers cannot secrete milk with α1-4 fucosylation and thus lack HMOs such as LNDFH I and II (12). Moreover, variation in HM from mothers diagnosed with preeclampsia (23), dependent on geographical origin (24, 25) and maternal obesity (26–28) has been observed.

Mothers are encouraged to exclusively breastfeed their infant for up to 6 months (29), but we know very less of the metabolic composition of HM over the course of prolonged lactation. The aims of this study were to identify and quantify metabolites in HM from term deliveries and elucidate the impact of lactation stage on HM metabolome from term delivery over 6 months of lactation at various time points. Here, we show that the changes in levels of particularly HMOs occur during the entire lactation period, with levels of 6-siallylactose (SL), LNT, and LNDFH I progressively declining at all lactation stages, and 3-FL gradually increasing. Moreover, we show that most changes are observed within the first month of lactation, but levels of gluconate, glutamine, and O-acetylcarnitine significantly vary between all lactation stages investigated, and levels of other metabolites, including 2-aminobutyrate, choline, ethanolamine, and O-phosphocholine, changed significantly after 1 or 2 months of lactation.

MATERIALS AND METHODS

Recruitment of Participants

The results presented in this study are a part of a larger cohort – INFAMILK, established in Cork, Ireland. The cohort seeks to characterize HM from mothers delivering birth at term over 25 weeks of lactation and further associate HM composition to infant gut microbial development. The subjects providing HM for this study were recruited from Cork University Maternity Hospital, Cork, Ireland, in the time period 2016–2020. Ethical approval for this study was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland. All participants enrolled in this study were healthy, provided written consent, and all relevant guidelines and regulations were followed.

Information on infant weight at birth, birth mode, gestational age (GA) at birth, infant sex, mother's pre-pregnancy body mass index (BMI), gravidae, and parity was recorded. All mothers included breastfed their infants exclusively.

Sample Collection

This sub-study comprises HM samples from 59 term deliveries. In total, 101 HM samples were collected from the 59 mothers at four different time points postpartum; weeks 1–2 (n = 27), 3–5 (n = 28), 7–9 (n = 31), and 20–25 (n = 15) postpartum.

Fresh human milk (10–20 ml) was expressed from mothers in a sterile container, stored at 4°C, and delivered to the laboratory within 24 h. Milk sub-samples were then split and frozen at −80°C immediately and shipped on dry ice to Aarhus University, Department of Food Science, where they were immediately placed in −80°C freezers until analysis.

^{1}H Nuclear Magnetic Resonance Spectroscopy Metabolomic Analysis of Human Milk

The 101 HM samples intended for ^{1}H nuclear magnetic resonance (NMR)-based metabolomics were processed in a random order as followed using standard protocol for milk-based metabolomics (17). Samples were thawed in water bath and kept on ice while Amplicon Ultra 0.5-ml 10-kDa spin filters (Millipore, Billerica, MA, United States) were being washed three times. The samples were skimmed by centrifugation at 4,000 g, at 4°C for 10 min, fat layer removed, and 500 μl of the skimmed milk transferred to individual Amplicon Ultra 0.5-ml 10 kDa spin filters. Next, the skimmed milk was filtered by centrifugation at 10,000 g at 4°C, for 30 min and 400 μl of filtered milk from each sample was transferred to an individual 5-mm NMR tube. In each NMR tube, 200 μl D_2O with 0.05% 3-(trimethylsilyl)propanoic acid (TSP, Sigma-Aldrich, Saint-Louis, MO, United States) was added. Spectra acquisition was acquired according to the study of Sundekilde et al. (17). Using a Bruker Avance III 600 spectrometer equipped with a 5-mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany), ^{1}H NMR spectra were acquired at 298 K and a ^{1}H frequency of 600.13 MHz. A single 90° pulse experiment (Bruker pulse sequence: noesypr1d) was run to acquire one-dimensional

spectra with a relaxation delay of 5 s. During the relaxation delay, water suppression was performed, and a total of 64 scans were comprised of 32.768 data points with a spectral width of 12.15 parts per million (ppm). The resulting ^{1}H NMR spectra were all referenced to TSP signal at 0 ppm. A line-broadening function by 0.3 Hz was applied to each ^{1}H NMR spectra, following by a Fourier transformation. Preprocessing of ^{1}H NMR spectra was subsequently conducted by phase and baseline corrections, done both automatically and manually using Topspin 3.2 (Bruker Biospin, Rheinstetten, Germany).

Identification of Metabolites and Statistical Analyses

Metabolites of the processed ^{1}H NMR spectra were identified using Chenomx NMR suite 9.0 (Chenomx Inc., Edmonton, AB, Canada) with Chenomx standard metabolite library and an in-house metabolite library.

Multivariate data analyses of the quantified metabolites were conducted using SIMCA 16 (MKS Data Analytics Solutions, Umea, Sweden). The dataset comprising the quantified metabolites was UV-scaled prior to the analysis. A principal component analysis (PCA) model was computed to evaluate overall variation in the dataset. Potential outliers were identified by inspecting the Hotellings T^2 plot with a cutoff point at 99%, and residuals plot DModX provided by SIMCA.

Univariate statistical analyses of quantified metabolites were conducted using R statistical environment[1] (version 4.0.3). Inter-group differences comprised grouping according to the week postpartum. If quantified metabolites adhered to normal distribution, analysis of variance (ANOVA) was conducted and inter-group differences analyzed *post hoc* by Tukey's honestly significant difference (HSD) test with a level of significance at $p < 0.05$. If quantified metabolites did not adhere to the normal distribution, inter-group differences were analyzed by the Kruskal–Wallis test following the Wilcoxon rank sum test with Benjamini–Hochberg false discovery rate corrected *p*-values with a level of significance at $p < 0.05$.

RESULTS

Subject Characteristics

The cohort consisted of 59 mothers delivering birth at term, of whom 30 provided samples at one time point throughout the study period, 18 provided two HM samples, nine provided three and two provided samples at all four time points (weeks 1–2, 3–5, 7–9, and 20–25, **Table 1**). As further presented in **Table 1**, the GA of the mothers was on average of 39.6 weeks, and they had an average BMI of 25.95 kg/m^2 and had given birth once before; 66% underwent spontaneous vaginal delivery, and 25.4% had Caesarean section, in line with population data from publicly-funded deliveries (30). The female/male infants' ratio was quite even, including about 47.5% females, and all infants were within the normal weight range at birth (2,500–4,500 g) (**Table 1**).

[1]http://cran.r-project.org

Lactation Stage Drives Overall Variation in the Human Milk Metabolome

We identified 54 different metabolites, including 19 amino acids and derivatives, 11 metabolites related to energy metabolism, 9 fatty acids and derivatives, and 12 sugar metabolites across the 101 samples analyzed. As secretor status of the mother influences the chemical structures of HMOs available in HM (20), mothers were identified as non-secretors or secretors dependent on the presence of the predominant resonance in 2-FL, an α1-2 fucosyl linkage (resonance signal at δ5.32 ppm). In total, 19% of mothers were classified as non-secretors. Further, Lewis status was identified dependent on the presence of an α1-4 fucosyl linkage in LNDFH I and II (resonance signal at δ5.03 ppm). Of the secretor mothers, six were identified as Lewis negative, corresponding to 10% of the total cohorts. We conducted a multivariate data analysis to analyze the overall variation in the metabolome of term HM by computing a PCA model. One of the samples had high levels of several amino acids (leucine, isoleucine, methionine, valine, phenylalanine, tyrosine, **Supplementary Figures 1A,B**) and, as this observation interfered with variation explained in PCA, the sample was excluded in the following analyses. The final model was summarized in six principal components (PCs) and described 57.4% of the variance in the data.

The first and second PC accounted for 32.4% of the total variation in the dataset and largely reflected variation over lactation from weeks 1–2 to 20–25. Samples of earlier stages of lactation clustered at the left side of the first PC, whereas samples of later stages of lactation clustered on the right side of the first PC, accounting for 17.8% of the variation in the data (**Figure 1A**). Samples from weeks 3–5 and 7–9 largely overlapped across the left and right sides of the first PC, indicating that discrimination between weeks 3–5 and 7–9 was not possible using the first two PCs (**Figure 1A**). The second PC mainly described some samples with high levels of lysine, leucine, isoleucine, and tyrosine as compared to the

TABLE 1 | Subject characteristics of cohort with continuous data presented as means, ± standard deviation and number of observations in round brackets.

Descriptive and clinical information	Cohort (59)
Mothers collecting samples at one/two/three/four different time-points (n)	30/18/9/2
GA at birth (weeks)	39.60 ± 0.98 (58)
Pre-pregnancy BMI (kg/m^2)	25.95 ± 4.95 (50)
BMI groups (NW/OW/OB) (n)	25/19/6 (50)
Delivery mode (C-section/SVD/Other) (n)	15/39/5 (59)
Gravida	2 ± 1.42 (59)
Parity	2 ± 0.93 (59)
Infant birth weight (g)	3674 ± 449 (58)
Infant sex (F/M)	28/31 (59)

Categorical data are presented as number of observations included in each category. Abbreviations: BMI; body mass index, C-section; Caesarean section, F; female, GA; gestational age, M; male, n; number of individuals, NW; normal weight, OB; obese, OW; overweight, SVD; spontaneous vaginal delivery.

FIGURE 1 | Principal component analysis of human milk samples (n = 100) from term deliveries collected from weeks 1 to 25 postpartum. **(A)** Scores scatter plot of observations collected colored according to sampling time postpartum. The legend denotes color-coded time points from weeks 1–2 to 20–25. **(B)** Corresponding loadings scatter plot of variables measured. Abbreviations: LDFT; lactodifucotetraose, LNDFH; lacto-N-difucohexaose, LNT; lacto-N-tetraose, SL; sialyllactose, W; week

remaining samples (**Figure 1B**). We found no influence of delivery mode, maternal pre-pregnancy BMI, gravida, parity, infant birth weight, or infant sex on the metabolome. Maternal secretor status largely separated the samples at the fifth PC, corresponding to approximately 6% of the total variation in the dataset (**Supplementary Figure 2A**).

As lactation stage was the largest source of variation in the data, we analyzed the corresponding loadings plot accordingly (**Figure 1B**). The vast majority of HMOs identified (2-FL, 3-SL, 6-SL, LNT, LNDFH I, lactodifucotetraose; LDFT) correlated with samples from earlier stages of lactation (weeks 1–2 and 3–5), whereas 3-FL correlated with later stages of lactation (**Figure 1B**). Moreover, citric acid, cis-aconitate, gluconate, ethanolamine, O-acetylcarnitine, pyruvate, and fucose correlated with samples from weeks 1–2, and lactose, caprate, caprylate, butyrate, glutamine, glutamate, glucose, 2-aminobutyrate, alanine, urea, and threonine correlated with samples from weeks 7–9 and 20–25 (**Figure 1B**).

Concentrations of Metabolites in Human Milk Change Across Multiple Lactation Stages

Coherently, to quantify the statistical importance of the relations observed in the multivariate data analysis, we analyzed the significant differences in the levels of metabolites between lactation stages by univariate analyses (**Tables 2–5**).

Concentrations of Sugars and Human Milk Oligosaccharides at Distinct Stages of Lactation

The metabolite detected in greatest concentrations at all stages of lactation was lactose, which significantly increased over lactation from 166.72 mM (57.07 g/L) at weeks 1–2 to 200.76 mM (68.72 g/L) in weeks 20–25 (**Table 2**). Regarding other sugars, glucose exhibited most variation in the first period after birth and significantly increased throughout the first month of lactation, from 0.94 mM in weeks 1–2 to 1.33 mM in weeks 3–5, though remained statistically stable thereafter. Levels of gluconate significantly decreased throughout all lactation stages, from 0.90 mM in weeks 1–2 to 0.25 mM in weeks 20–25 (**Table 2**).

In general, the HMOs detected in highest levels were 2-FL, 3-FL, and LNT, although they exhibited distinctive changes over the investigated 1–25 weeks of lactation (**Table 2**). Levels of 2-FL, 3-SL, 6-SL, LDFT, LNDFH I, and LNT were all significantly higher early in lactation. Specifically, 2-FL was significantly more abundant in milk from weeks 1–2 (2.48 mM, 1.21 g/L) compared to all other lactation stages, but levels were not significantly different when comparing between weeks 3–5, 7–9, and 20–25 (**Table 2**). Levels of 3-SL were significantly more abundant in weeks 1–2 (0.18 mM, 0.12 g/L) compared to all other lactation stages, and between weeks 3–5 (0.147 mM, 0.096 g/L) weeks 7-9 (0.12 mM, 0.08 g/L), the levels significantly decreased. Levels of 6-SL, LNDFH I, and LNT significantly and progressively decreased over the entire investigated period, from 0.95 mM (0.62 g/L 6-SL), 1.19 mM (1.19 g/L, LNDFH I), and 3.82 mM (2.25 g/L, LNT) in weeks 1–2 to 0.08 mM (0.05 g/L, 6-SL), 0.40 mM (0.40 g/L, LNDFH I), and 1.13 mM (0.80 g/L, LNT) in weeks 20–25 (**Table 2**). In contrast, 3-FL was dominant in later stages of lactation and significantly increased in levels from the first weeks following birth (0.94 mM, 0.46 g/L in weeks 1–2) to later stages of lactation (2.61 mM, 1.27 g/L in weeks 20–25, **Table 2**).

Concentrations of Amino Acids and Derivatives at Distinct Stages of Lactation

Glutamate was detected in the highest concentration out of all free amino acids identified and increased significantly during the first month of lactation from 0.89 (weeks 1–2) to 1.25 mM (week 3.5). Whereas levels of glutamate changed insignificantly after 1 month of lactation, levels of glutamine increased progressively and significantly at all time points from 0.07 mM in weeks 1–2 to 0.59 mM in weeks 20–25 (**Table 3**). Similarly, levels of O-acetylcarnitine significantly decreased throughout all lactation stages from 0.04 mM in weeks 1–2 to 0.01 mM in weeks 20–25 (**Table 3**).

TABLE 2 | Mean levels (in mM ± standard deviation) of sugars and human milk oligosaccharides in human milk from term deliveries sampled weeks 1–2, 3–5, 7–9, and 20–25 postpartum.

Sugars and HMOs	Week 1–2 (mM ± SD)	Week 3–5 (mM ± SD)	Week 7–9 (mM ± SD)	Week 20–25 (mM ± SD)
2-FL*	$2.48^{a} \pm 0.76$	$1.83^{b} \pm 0.78$	$1.76^{b} \pm 0.63$	$1.31^{b} \pm 0.43$
3-FL	$0.94^{a} \pm 0.83$	$1.57^{b} \pm 1.04$	$2.15^{bc} \pm 1.88$	$2.61^{c} \pm 1.13$
3-SL	$0.18^{a} \pm 0.05$	$0.15^{b} \pm 0.05$	$0.12^{c} \pm 0.04$	$0.12^{bc} \pm 0.03$
6-SL*	$0.95^{a} \pm 0.25$	$0.55^{b} \pm 0.18$	$0.33^{c} \pm 0.14$	$0.08^{d} \pm 0.04$
Fucose	0.22 ± 0.15	0.16 ± 0.16	0.16 ± 0.11	0.20 ± 0.08
Gluconate	$0.90^{a} \pm 0.50$	$0.60^{b} \pm 0.29$	$0.44^{c} \pm 0.30$	$0.25^{d} \pm 0.21$
Glucose*	$0.94^{a} \pm 0.39$	$1.33^{b} \pm 0.43$	$1.49^{b} \pm 0.52$	$1.70^{b} \pm 0.40$
Lactose*	$166.72^{a} \pm 16.08$	$180.98^{b} \pm 15.74$	$188.56^{bc} \pm 15.41$	$200.76^{c} \pm 9.95$
LDFT	$0.68^{a} \pm 0.75$	$0.53^{ab} \pm 0.61$	$0.41^{b} \pm 0.43$	$0.38^{ab} \pm 0.14$
LNDFH I	$1.19^{a} \pm 0.43$	$0.95^{b} \pm 0.33$	$0.70^{c} \pm 0.29$	$0.40^{d} \pm 0.19$
LNT	$3.82^{a} \pm 1.26$	$2.75^{b} \pm 1.07$	$1.96^{c} \pm 0.83$	$1.13^{d} \pm 0.54$
Myo-inositol	0.78 ± 0.21	0.74 ± 0.24	0.82 ± 0.26	0.66 ± 0.24

*Significant differences were analyzed by one-way ANOVA followed by Tukey's HSD with a level of significance at $p < 0.05$. If variables were not normally distributed, statistical significance between time points was analyzed by the Kruskal–Wallis test following the Wilcoxon rank sum test with Benjamini–Hochberg corrected p-values and a level of significance at $p < 0.05$. Significant differences between time points are indicated by different letters. Metabolites that significantly differ over lactation are written in bold. Abbreviations: FL; fucosyllactose, HMOs; human milk oligosaccharides, LDFT; lactodifucotetraose, LNDFH; lacto-N-difucohexaose, LNT; lacto-N-tetraose, mM; millimolar, SD; standard deviation, SL; sialyllactose, *variables normally distributed.*

TABLE 3 | Mean levels (in mM ± standard deviation) of amino acids and their derivatives in human milk from term deliveries sampled weeks 1–2, 3–5, 7–9, and 20–25 postpartum.

Amino acids and derivatives	Week 1–2 (mM ± SD)	Week 3–5 (mM ± SD)	Week 7–9 (mM ± SD)	Week 20–25 (mM ± SD)
2-aminobutyrate	$0.01^{a} \pm 0.00$	$0.01^{a} \pm 0.01$	$0.01^{b} \pm 0.01$	$0.02^{c} \pm 0.01$
Alanine	$0.18^{a} \pm 0.12$	$0.25^{b} \pm 0.09$	$0.24^{b} \pm 0.08$	$0.26^{b} \pm 0.05$
Aspartate	0.05 ± 0.04	0.06 ± 0.03	0.06 ± 0.02	0.08 ± 0.07
Betaine	$0.06^{a} \pm 0.01$	$0.07^{ab} \pm 0.02$	$0.07^{ab} \pm 0.01$	$0.08^{b} \pm 0.01$
Carnitine	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.03
Glutamate*	$0.89^{a} \pm 0.57$	$1.25^{b} \pm 0.43$	$1.45^{b} \pm 0.48$	$1.51^{b} \pm 0.32$
Glutamine	$0.07^{a} \pm 0.05$	$0.23^{b} \pm 0.13$	$0.41^{c} \pm 0.24$	$0.59^{d} \pm 0.23$
Glycine*	0.58 ± 0.12	0.58 ± 0.08	0.61 ± 0.14	0.65 ± 0.15
Isoleucine	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
Leucine	$0.02^{a} \pm 0.02$	$0.03^{ab} \pm 0.02$	$0.03^{b} \pm 0.01$	$0.03^{b} \pm 0.01$
Lysine	$0.04^{a} \pm 0.05$	$0.02^{b} \pm 0.01$	$0.02^{b} \pm 0.01$	$0.02^{b} \pm 0.01$
Methionine	$0.01^{a} \pm 0.01$	$0.01^{b} \pm 0.00$	$0.01^{b} \pm 0.00$	$0.01^{b} \pm 0.00$
O-acetylcarnitine	$0.04^{a} \pm 0.02$	$0.02^{b} \pm 0.02$	$0.01^{c} \pm 0.01$	$0.01^{d} \pm 0.00$
Phenylalanine	$0.01^{a} \pm 0.01$	$0.01^{ab} \pm 0.01$	$0.01^{b} \pm 0.01$	$0.01^{ab} \pm 0.00$
Taurine	0.30 ± 0.11	0.26 ± 0.12	0.23 ± 0.07	0.26 ± 0.13
Threonine	$0.07^{a} \pm 0.05$	$0.08^{a} \pm 0.04$	$0.10^{b} \pm 0.04$	$0.11^{b} \pm 0.05$
Tyrosine	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Urea	1.62 ± 0.63	1.71 ± 0.89	1.76 ± 0.88	2.01 ± 1.12
Valine	$0.04^{a} \pm 0.04$	$0.06^{b} \pm 0.02$	$0.06^{b} \pm 0.02$	$0.05^{b} \pm 0.01$

*Significant differences analyzed by one-way ANOVA followed by Tukey's HSD with a level of significance at $p < 0.05$. If variables were not normally distributed, statistical significance between time points was analyzed by the Kruskal–Wallis test following the Wilcoxon rank sum test with Benjamini–Hochberg corrected p-values and a level of significance at $p < 0.05$. Significant differences between time-points are indicated by different letters. Metabolites that significantly differ over lactation are written in bold. Abbreviations: mM; millimolar, SD; standard deviation, *variables normally distributed.*

TABLE 4 | Mean levels (in mM ± standard deviation) of fatty acids and derivatives in human milk from term deliveries sampled weeks 1–2, 3–5, 7–9, and 20–25 postpartum.

Fatty acids and derivatives	Week 1-2 (mM ± SD)	Week 3-5 (mM ± SD)	Week 7-9 (mM ± SD)	Week 20-25 (mM ± SD)
Acetate	0.02 ± 0.01	0.04 ± 0.10	0.01 ± 0.01	0.02 ± 0.01
Butyrate	$0.01^{a} \pm 0.02$	$0.04^{b} \pm 0.04$	$0.08^{b} \pm 0.09$	$0.18^{c} \pm 0.17$
Caprate	$0.06^{a} \pm 0.06$	$0.09^{ab} \pm 0.08$	$0.09^{ab} \pm 0.06$	$0.12^{b} \pm 0.06$
Caprylate	$0.08^{a} \pm 0.08$	$0.13^{ab} \pm 0.11$	$0.15^{bc} \pm 0.12$	$0.25^{c} \pm 0.17$
Choline	$0.14^{a} \pm 0.07$	$0.13^{a} \pm 0.09$	$0.16^{a} \pm 0.11$	$0.23^{b} \pm 0.08$
Ethanolamine	$0.08^{a} \pm 0.02$	$0.07^{a} \pm 0.02$	$0.06^{b} \pm 0.02$	$0.06^{b} \pm 0.02$
O-phosphocholine	$0.67^{a} \pm 0.20$	$0.60^{a} \pm 0.21$	$0.50^{b} \pm 0.18$	$0.31^{c} \pm 0.15$
Sn-glycero-3-phosphocholine*	0.49 ± 0.23	0.49 ± 0.23	0.43 ± 0.15	0.49 ± 0.16
Valerate	$0.02^{a} \pm 0.02$	$0.05^{b} \pm 0.05$	$0.06^{b} \pm 0.06$	$0.15^{c} \pm 0.15$

*Significant differences analyzed by one-way ANOVA followed by Tukey's HSD with a level of significance at $p < 0.05$. If variables were not normally distributed, statistical significance between time points was analyzed by the Kruskal–Wallis test following the Wilcoxon rank sum test with Benjamini–Hochberg corrected p-values and a level of significance at $p < 0.05$. Significant differences between time-points are indicated by different letters. Metabolites that significantly differ over lactation are written in bold. Abbreviations: mM; millimolar, SD; standard deviation, *variables normally distributed.*

As in the case of glutamate, other amino acids likewise changed significantly throughout the first month of lactation only. Alanine and valine levels significantly increased during the first month of lactation from 0.18 mM (alanine) and 0.04 mM (valine) at weeks 1–2 to 0.25 mM (alanine) and 0.06 mM (valine) at weeks 3–5. In contrast, lysine decreased the first month of lactation from 0.04 mM to 0.02 mM and likewise remained stable from weeks 3–5 and onward (**Table 3**). Finally, levels of other amino acids were constant throughout the first month of lactation and only changed significantly after weeks 3–5. This included levels of threonine, leucine, and 2-aminobutyrate. Levels of threonine were constant throughout the first month of lactation and increased in levels from weeks 1–2 and 3–5 to 7.9 week (0.10 mM). Likewise, levels of leucine were significantly increased when comparing weeks 1–2 (0.02 mM) and 7–9 (0.03 mM) or 20–25 week (0.03 mM). Levels of 2-aminobutyrate were constant at the first month of lactation from weeks 1–2 to 3–5 at a level of 0.010 mM and thereafter increased progressively and significantly to 0.021 mM in weeks 20–25, whereas levels of betaine were significantly increased when comparing weeks 1–2 (0.06 mM) and 20–25 (0.08 mM) only.

Concentrations of Fatty Acids and Derivatives at Distinct Stages of Lactation

Regarding the fatty acids, butyrate (0.18 mM), caprate (0.12 mM), caprylate (0.25 mM), and valerate (0.15 mM) were detected in highest levels in late lactation at weeks 20–25 (**Table 4**). When comparing weeks 1–2 and 7–9 and/or 20–25, levels of caprate and

TABLE 5 | Mean levels (in mM ± standard deviation) of metabolites related to energy metabolism in human milk from term deliveries sampled weeks 1–2, 3–5, 7–9, and 20–25 postpartum.

Energy metabolites	Week 1–2 (mM ± SD)	Week 3–5 (mM ± SD)	Week 7–9 (mM ± SD)	Week 20–25 (mM ± SD)
2-oxoglutarate	$0.04^{a} \pm 0.02$	$0.05^{ab} \pm 0.03$	$0.05^{ab} \pm 0.03$	$0.07^{b} \pm 0.06$
Acetate	0.02 ± 0.01	0.04 ± 0.10	0.01 ± 0.01	0.02 ± 0.01
Cis-Aconitate*	$0.01^{a} \pm 0.00$	$0.01^{b} \pm 0.00$	$0.01^{b} \pm 0.00$	$0.01^{b} \pm 0.00$
Citric acid*	$4.17^{a} \pm 1.00$	$2.98^{b} \pm 0.80$	$2.58^{b} \pm 0.71$	$1.88^{c} \pm 0.57$
Creatine*	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.03
Creatinine*	$0.05^{ab} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.04^{b} \pm 0.01$
Creatine phosphate	$0.02^{a} \pm 0.01$	$0.02^{a} \pm 0.02$	$0.02^{a} \pm 0.02$	$0.01^{b} \pm 0.01$
Fumarate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lactate	0.12 ± 0.07	0.13 ± 0.10	0.16 ± 0.33	0.08 ± 0.04
Pyruvate	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
Succinate	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Unclassified metabolites				
2-hydroxybutyrate	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Hippurate	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
Pantothenate	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

*Significant differences analyzed by one-way ANOVA followed by Tukey's HSD with a level of significance at $p < 0.05$. If variables were not normally distributed, statistical significance between time points was analyzed by the Kruskal–Wallis test following the Wilcoxon rank sum test with Benjamini–Hochberg corrected p-values and a level of significance at $p < 0.05$. Significant differences between time points are indicated by different letters. Metabolites that significantly differ over lactation are written in bold. Abbreviations: mM; millimolar, SD; standard deviation, *variables normally distributed.*

caprylate were significantly increased, and levels of butyrate and valerate increased during the first month of lactation (weeks 1–2 to 3–5) and then remained stable before significantly increasing in weeks 20–25.

Moreover, O-phosphocholine levels were unchanged during the first month of lactation (0.67–0.60 mM in weeks 1–2 to 3–5), but then decreased significantly at lactation stage week 7–9 (0.50 mM) and weeks 20–25 (0.31 mM, **Table 4**). Similarly, levels of ethanolamine did not significantly vary throughout the first month of lactation (weeks 1–2 vs. 3–5) but decreased significantly at weeks 7–9 (0.06 mM), after which the levels again were statistically stable (0.06 mM at weeks 20–25, **Table 4**). Levels of choline were statistically constant until weeks 7–9 (0.16 mM), after which the levels significantly increased to weeks 20–25 of lactation (0.23 mM).

Concentrations of Energy Metabolites and Unclassified Metabolites at Distinct Stages of Lactation

We observed that citric acid, pyruvate, cis-aconitate, fumarate, and succinate all tended to cluster on the left side of the first PC in the PCA model and thus correlated with samples from earlier stages of lactation (**Figures 1A,B**). Based on univariate data analysis, citric acid levels significantly decreased over lactation from 4.17 mM in weeks 1–2 to 1.88 mM in weeks 20–25 (**Table 5**). Cis-aconitate was detected in highest level at weeks 1–2 (0.01 mM) and decreased significantly in the first month of lactation, weeks 3–5, after which the levels did not significantly change from weeks 3–5 to 20–25 (**Table 5**). Neither pyruvate, fumarate nor succinate changed significantly throughout lactation, whereas 2-oxoglutarate increased toward the late lactation from 0.04 mM in weeks 1–2 to 0.07 mM in weeks 20–25 (**Table 5**). Finally, levels of creatinine were significantly increased at weeks 3–5 and 7–9 (0.56 mM at both time points) compared to weeks 20–25 (0.40 mM), whereas levels of creatinine at weeks 1–2 were not significantly different from the remaining lactation stages (**Table 5**).

DISCUSSION

Human milk is nutritionally both a highly complex and complete food provided for infants to ensure optimal development early in life, and besides from macronutrients consists of bioactive compounds and metabolites deriving from maternal metabolism. The maturation of analytical technology in metabolomics is still ongoing (31). However, data acquired using different technologies should be comparable if absolute quantitation of metabolites is performed. NMR has previously been a widely used technique in milk-based metabolomic studies, as NMR offers absolute quantitation and measures the most abundant metabolites in the μm range and up (16, 17). However, care must be taken when comparing across studies as several parameters might influence the milk metabolome, where sample collection is most prominent (13). The most dominant sampling technique is spot sampling, where a small amount of fore or hind milk is collected. Less common is complete emptying of the breast, as this might interfere with the mother's natural breastfeeding of the baby. The composition of HM is known to vary over lactation moving from colostrum to mature milk (around 1 month after birth), though less is known about the composition of HM metabolome from clinically healthy mothers delivering at term throughout lactation, beyond 1 month postpartum. Overall, we find that the metabolome of HM continuously varies throughout lactation and some changes are distinctive for milk excreted after 1 to 2 months of lactation. The trend for lactose,

medium- and short-chain fatty acids, such as butyrate, caprate, and caprylate, the amino acids glutamate and glutamine to positively correlate with progressing lactation, and citric acid and HMOs to negatively correlate with progressing lactation is in accordance with similar tendencies observed by others (15–17, 23, 32). Univariate data analysis largely substantiated the tendencies observed in the multivariate data analysis. Levels of gluconate, citric acid, cis-aconitate, ethanolamine, and O-acetylcarnitine were significantly higher early in lactation than in later stages. In contrast, levels of lactose, glucose, glutamate, valine, valerate, threonine, alanine, glutamine, butyrate, 2-aminobutyrate, caprate, and caprylate were found to significantly increase from weeks 1–2 to later stages of lactation.

Sugars and Human Milk Oligosaccharides

Compared to the works of others, lactose has been observed to significantly increase over lactation in HM from term deliveries over 1 month of lactation (16) and in HM sampled up to 3 months postpartum (15). The study reported by Spevacek et al. (16) used milk collected by pumping a full breast, whereas Andreas et al. (15) predominantly collected hind milk, and fore milk is collected in this study. Despite the differences in collection strategy, there is an agreement of the results and also confirmed here. Glucose changed in the levels in accordance with previous reports (16), although it remained stable after 1 month of lactation. In another study, significant increases in levels of glucose were only observed throughout the first 10 days postpartum, but not at later stages of lactation (6–10 vs. + 10 days, latter spanning up to 3 months postpartum) (15). Gluconate is an oxidized form of glucose and, to the best of our knowledge, no other metabolomic studies have to date made similar observations. One study observed a positive correlation between gluconate and week 4 of lactation (n = 9) compared to week 1 (n = 10) (33), and another reported no correlation with lactation stage (17).

Human milk oligosaccharides particularly varied throughout lactation, as observed both from the multivariate data analysis and the univariate data analysis. Human milk oligosaccharides are acknowledged prebiotic components of HM (34), with high structural diversity (21), and their concentrations have been reported to be dependent on maternal phenotype (20, 35, 36), term- or preterm delivery (17), maternal diet (37), and lactation stage (38). Maternal phenotype specifically relates to secretor status and Lewis gene type of the mother, which determine the chemical composition of fucosylated HMOs in the milk. In line with other findings, 19% of mothers were classified as non-secretors (16, 39), and of the secretor mothers, six were identified as Lewis negative, corresponding to 10% of the total cohort, and none were non-secretors and Lewis negative, consistent with the expectation for this phenotype to be present in 1% of the population (39). We found that secretor status mainly discriminated observations along the fifth PC in a multivariate data analysis, thus only contributing to the variation and composition of HM metabolites in minor degree. Generally, other studies have observed a greater contribution of secretor status on the metabolome of HM (16, 17). However, we observed clear trends in terms of changes in levels of HMOs over lactation. Levels of all HMOs except 3-FL were significantly higher in early stages of lactation. This trend is in line with previous results (38). Multiple interactions between HMOs and the infant gut microbiota and environment have been extensively reviewed previously (6, 34, 40). Research has suggested that 2-FL and 3-FL are negatively correlated (38), and studies have demonstrated that both 2-FL and 3-FL promote the growth of infant gut symbionts, enhance production of short chain fatty acids, and improve intestinal mobility and epithelial barrier function (6). Thus, despite overall decreasing levels of HMOs identified in this study over lactation and the lack of α1-2 fucosylated HMOs in HM from non-secretor mothers, increasing levels of 3-FL over lactation provide continuous bioactivity to the neonatal gut environment. In addition to variation dependent on lactation stage, we observed a tendency for maternal secretor status to affect the concentration of 3-FL, LNT, and 3-SL in HM, whereby non-secretor mothers tended to have higher concentrations of these HMOs in their milk. Although we did not conduct statistical analyses on these observations owing to small sample size of non-secretor mothers, the tendency for 3-FL and LNT to be increased in HM from non-secretor mothers delivering birth at term has been observed elsewhere (36, 41).

Amino Acids

Glutamine and glutamate are generally present in highest concentration out of all free amino acids in HM (42), and both glutamine and glutamate have previously been observed in metabolomic studies to significantly increase over or positively correlate with lactation (15–17). However, contrary to the results presented in this study, Andreas et al. (15) found glutamine levels to significantly increase between the first 5 days of lactation and + 10 days of lactation, but not when comparing lactation day 6–10 with + 10 days using a multi-omics approach (n = 57). The results presented in the study of Spevacek et al. (16) conducting ^{1}H NMR metabolomics on HM from a cohort of term deliveries (n = 15) collected at days 0–5 (colostrum), 14 (transitional HM), and 28 (mature HM) indicate that both glutamine and glutamate significantly increase over the first month of lactation, consistent with the present findings. Glutamine and glutamate are important for growth of the neonate's gut as enterocytes of the neonate utilize these amino acids for metabolism leading to improved barrier function. As such, glutamine is utilized in the intestine and converted to alanine, during which NADH and $FADH_2$ are generated, providing energy for gut enterocytes (18). Adding to this, both amino acids interact with the immune system and gut microbiota (43). Moreover, glutamine is widely involved in various metabolic pathways in the human body, including the regulation of the immune system and cell functions (44). The increased concentration of these amino acids over lactation could facilitate gut maturation and growth of the infant by providing proteinogenic amino acids readily involved in protein synthesis (43, 44). In support of this, a study observed significantly increased glutamate levels and increased difference in glutamine levels from 1 to 4 months in HM for infants with faster weight gain in the first 4 months

postpartum (45). Other amino acids significantly changed in levels over lactation, of which some significantly changed the first month of lactation only (alanine, valine, methionine, and lysine), whereas others were constant at the first month of lactation and changed significantly with progressing lactation afterward (threonine, leucine, 2-aminobutyrate). Alanine, valine, lysine, and methionine showed similar trends in the present univariate data analysis as described by others (15, 16, 46), though, in the multivariate data analysis, valine was not strongly correlated with later stages of lactation. Moreover, whereas the levels of threonine were constant throughout the first month of lactation, in line with previous observations (16), we find that after weeks 3–5, levels of threonine increased significantly, which is not in line with previous observations (46).

Valine, methionine, lysine, leucine, threonine, and phenylalanine are all indispensable amino acids for the new-born baby (46). In the mammary gland, a proportion of branched-chain amino acids (BCAAs) is catabolized for synthesis of glutamate and glutamine (47), which increased over lactation. The proportion of BCAAs excreted in milk is important for infant growth and development early in life, as they provide nitrogen for protein synthesis, neurotransmitter synthesis and interact with the immune system (48, 49). In support of this, a study observed that BCAAs and insulin-trophic amino acids were associated with preterm infants experiencing fast growth at the first month postpartum (50), thereby underlining the potential importance of particularly BCAAs for infant growth early in lactation.

We found higher levels of 2-aminobutyrate after 7–9 weeks of lactation, although another study observed significantly higher levels of 2-aminobutyrate the first month of lactation in HM from term deliveries (16), and abundance of 2-aminobutyrate was significantly increased throughout the first 6 months of lactation compared to after 6 months of lactation (n = 130) in a third study (51). Although not fully in agreement with this study, the aforementioned results, combined with the ones in the present study, demonstrate that, throughout lactation and between lactation stages, 2-aminobutyrate is among the metabolites continuously changing in concentration, potentially reaching a maximum around the first 6 months of lactation. 2-aminobutyrate is a non-proteinogenic amino acid involved in catabolism of methionine, threonine, and serine. Associations have been observed between lower levels of 2-aminobutyrate in HM and inflammatory bowel disease for mothers (52), and higher levels in HM have been reported for overweight/obese mothers at 1 month of lactation (26).

Additionally, levels of O-acetylcarnitine changed significantly throughout all the stages of lactation. This trend is in accordance with the results of others (16). The implications of O-acetylcarnitine (acetyl-L-carnitine) in HM are not well characterized. In the cells of the human body, O-acetylcarnitine is formed from the transfer of an acetyl-group to carnitine (53). Both carnitine and O-acetylcarnitine are involved in fatty acid oxidation, by which the carnitine shuttle facilitates the transport of acyl-CoA derivatives of long-chain fatty acids across the mitochondrial membrane for oxidation (54). Various species of acylcarnitines have been reported to be implicated in insulin sensitivity, with long chain acylcarnitines specifically being indicative of development of type 2 diabetes, and increased levels of acylcarnitines from BCAA metabolism being reported in HM from overweight/obese mothers (26, 54, 55). However, acetylcarnitine has been associated with improvement of glucose tolerance, metabolic flexibility, and neurological function (53, 56, 57). How levels of O-acetylcarnitine in HM might affect infant metabolism is unresolved although, as an acetylated form of carnitine, O-acetylcarnitine is readily absorbed in the gut (53), with the potential of assisting uptake of carnitine in the infants. Despite no observed significant change in levels of carnitine over lactation in this study, changing levels of O-acetylcarnitine over lactation could reflect shifts in metabolic activity of the mammary gland involving glucose metabolism and fatty acid oxidation.

Compared to the works of others, betaine, leucine, and phenylalanine have been found to differ from that presented in this study (16, 17, 46). Levels of betaine were significantly increased when comparing weeks 1–2 and 20–25 only, whereas others have found decreased levels of betaine over lactation (16, 17). Research to date suggests that multiple factors influence the composition of free amino acids in HM besides the lactation stage. As such, variation in amino acid content of HM has been observed to depend on the infant sex (45) and maternal BMI (28). Moreover, variations in amino acid composition within a single feed have been observed, with increased levels of free amino acids, phenylalanine, threonine, alanine, valine, glutamine, and serine in foremilk (58).

Fatty Acids and Derivatives

The fatty acids detected in this study using ^{1}H NMR spectroscopy – butyrate, caprate, caprylate, and valerate all significantly increased over lactation and were found to be positively correlated with samples of late lactation in a multivariate data analysis. Meng et al. investigated macronutrient levels and fatty acid profiles in HM from same cohort as this study and found a tendency for both caprate and caprylate to increase over 24 weeks of lactation (5.51 to 6.21 mg g^{-1} and 1.83 to 2.06 mg g^{-1} respectively), though the results were not significant. However, a significant impact of maternal BMI on levels of saturated fatty acids, including caprylate, and an additional effect of infant gender on levels of caprylate in HM at 24 weeks of lactation were identified[2]. In another cohort studying HM from mothers delivering term (n = 30), spanning from colostrum to mature milk (comprising milk sampled once at 5–33 weeks of lactation) butyrate, caprate, and caprylate correlated positively with mature milk (17), in line with the present results. Increased levels of short- and medium-chain fatty acids over lactation have been suggested to be related to mammary gland maturation and coherently upregulated *de novo* fatty synthesis (59). A recent review highlighted how medium-chain fatty acids affect immune system processes and gut microbiota (60) over and above serving as an energy source. Concentrations of butyrate, caprate, and caprylate have been reported to increase over lactation in other

[2]Meng F, Uniacke-Lowe T, Lanfranchi E, Meehan G, O-Shea CA, Dennehy T, et al. *A Longitudinal Study of Macronutrients Levels, Plasmin Activity, and Fatty Acid Profile in Human Milk*. (personal communication, manuscript in preparation).

studies (8, 17, 61). Furthermore, the influence of maternal diet has been investigated in an intervention study (n = 14), which indicated that a low-fat diet results in higher concentrations of medium-chain fatty acids (62).

Levels of valerate followed the same pattern over lactation as described for butyrate, caprate, and caprylate, although no other studies investigating HM metabolome have reported on valerate. The origin or importance of valerate in HM is not understood, but others highlight valerate produced by the gut microbiota to induce growth of intestinal epithelium and protection against diseases, such as colitis and cardio-metabolic diseases (63). It is possible that valerate detected in our samples derives from microbial metabolism by species of the HM microbiome (64), which change significantly from birth to 24 weeks of age (65).

In agreement with the present results, others found the levels of O-phosphocholine to be constant throughout the first month of lactation (16) and decrease significantly after 1-month of lactation in a pilot study using a single donor (32). In contrast, results from another cohort sampling HM at 3 days and 6 months postpartum (n = 31) observed the opposite trend for O-phosphocholine (23) and increasing levels of ethanolamine over 1 month of lactation and decreasing levels of choline have been observed (16). Large inter-individual variability in HM content has been reported for levels of ethanolamine and O-phosphocholine dependent on regional differences (25), and in choline content of HM between mothers, as free choline in HM was found to be correlated with levels of free choline, phospholipid-bound choline and glycerophosphocholine in the maternal serum (19). O-phosphocholine, choline, and ethanolamine in HM derive from the milk fat globule membrane, as these are common head groups for phospholipids, but also found as free components in the milk (66, 67). While ethanolamine is synthesized into phosphatidylethanolamine, O-phosphocholine and choline are intermediates in the synthesis of phosphatidylcholine (66, 68).

Metabolites Related to Energy Metabolism

Of metabolites related to energy metabolism, we observed that citric acid, pyruvate, cis-aconitate, fumarate, and succinate all tended to cluster on the left side of the first PC in the multivariate data analysis and thus correlated with samples from earlier stages of lactation. Of these, the univariate data analysis reflected significant variation in levels of citric acid, cis-aconitate, and 2-oxoglutarate over lactation. As mentioned previously, other studies have identified increased levels of citric acid in earlier stages of lactation compared to later stages of lactation (15, 16, 32, 51), though some discrepancies exist in terms of variation in metabolites related to energy metabolism found in HM. In a study conducting HM metabolomics across various geographical regions with HM sampled at 1 month of lactation (n = 109), 2-oxoglutarate, citric acid, and lactose were positively correlated, although with region-specific variation in levels of citric acid and 2-oxoglutarate (22). In the study by Spevacek et al. (16), levels of 2-oxoglutarate decreased throughout the first month of lactation, opposite to what was observed in this study. Moreover, fumarate and pyruvate levels significantly decreased over 1 month of lactation (16), whereas neither levels of cis-aconitate nor citric acid significantly changed, in contrast to the present results (**Table 5**), and results of others (15, 17). Citric acid, cis-aconitate, fumarate, succinate, pyruvate, and 2-oxoglutarate are all associated with tricarboxylic acid (TCA) cycle (69), and correlations could reflect the metabolic activity of the mammary gland. In a study comparing the metabolome of the tissue of mammary gland, milk, rumen fluid, and urine from lactating and non-lactating cows, TCA cycle was particularly upregulated in mammary gland of lactating cows based on pathway analysis, with citric acid identified as a biomarker of lactation and expressed in greater levels (70). 2-oxoglutarate is a metabolite involved in both carbon metabolism and nitrogen metabolism, serving as an intermediate of TCA cycle and an intermediate in nitrogen-assimilatory reactions, respectively (71). Thus, the increased levels of 2-oxoglutarate observed in this study might likewise partly be linked to glutamate synthesis, as both glutamine and glutamate were observed to be increased in later stages of lactation. Overall, higher levels of citric acid and cis-aconitate early in lactation, lower levels of 2-oxoglutarate, and increased levels of glutamine and glutamate with progressing lactation were observed. As partly implied by Smilowitz et al. (36), part of these correlations could be explained by the hypothesis that, later in lactation, citric acid and potentially cis-aconitate are partly being utilized for 2-oxoglutarate production in the mammary gland and eventually glutamate synthesis, thus providing increased nitrogen load for the infant for amino acid synthesis.

Discrepancies Between Analyses and Limitations of Study

We found some discrepancies in the tendencies reflected by the multivariate data analysis compared to the univariate data analysis. Specifically, levels of 2-oxoglutarate, choline, and O-phosphocholine changed significantly over lactation in the univariate analysis, but this tendency was not evident in the PCA model, as the metabolites have a low impact on the first PC. On the other hand, pyruvate and fucose seemed to correlate with samples from earlier stages of lactation in the PCA model and urea with later stages of lactation, but the levels were not significantly different in univariate data analysis. Finally, creatine phosphate decreased significantly over lactation when comparing weeks 20–25 with the remaining lactation stages but, in the multivariate data analysis, creatine phosphate correlated with samples from later stages of lactation on the first PC. However, this correlation could possibly reflect correlation with samples from weeks 3–5 to 7–9 as these largely overlapped in the PCA. Part of these discrepancies between the multivariate and univariate analysis may be ascribed to the fact that the PCA model comprised 57.4% of the variation in the dataset, and lactation stage explained about 33% of the variation in the data, leaving some residual variation not accounted for in the multivariate analysis. Furthermore, PCA models also take co-variance into consideration, unlike univariate analyses which do not. Moreover, in the multivariate data analysis, some observations strongly correlated with free amino acids including

tyrosine, isoleucine, leucine, and lysine. These did not derive from the same donor nor consistently the same time point, and the reason for the similarities of these samples in terms of levels of lysine, leucine, isoleucine, and tyrosine cannot be explained with the information available.

A strength of this study is the inclusion of multiple time points postpartum, and the duration of the study moving beyond 1 month of lactation to 6 months of lactation. This enables analysis of changes in HM beyond the state of maturity and at various lengths of lactation. Previous studies have investigated variation over lactation in HM from term mothers longitudinally (1, 16, 23, 26, 27, 52, 72, 73), although the research focus has included mothers delivering term with pathological conditions (23, 52, 72), inclusion of samples from various lactation stages ranging from 1 week postpartum to 6 months, and with maximum three HM collections throughout the study period. For example, one study collected HM at 3 days and 6 months postpartum (23), one collected after 1 month and then again at 6 months (26), three studies sampled three times throughout the first month after birth (16, 72, 73), one after 3 and 6 months (52), and one study collected the first milk (colostrum), mature milk (2 months postpartum), and milk at 6 months (27). Thereby, this study provides insights into the HM metabolome of mature milk, and we demonstrate that even mature milk progressively varies over lactation with specific changes in HM metabolome not observed in HM from 1 month of lactation. Limitations of this study are the limited ethnicity and geographical region of included participants. The cohort is based on Irish women, and so the results presented could be influenced by geographical differences (25). Moreover, several participants did not deliver milk samples at all time points, by which these observations were single-point observations rather than longitudinal, and we have not collected samples between 9 weeks of lactation and 20 weeks of lactation, leaving a gap in terms of understanding the development of HM metabolome within this timeframe. Evidently, our results are restricted by the limitations of our chemical analysis, as ^{1}H NMR-based metabolomics has a high lower limit of detection in the μm range than MS-based methods (nM range) and mainly detects constituents in the aqueous phase, such as amino acids, saccharides, and energy metabolites, but cannot detect the full range of lipid species found in HM (15, 74). Nevertheless, the results of this study add to the knowledge of the development of the HM metabolome from term deliveries in the first period after birth from 1 week to about 6 months postpartum. Moreover, we comprehensively characterize HM composition from macronutrient level to metabolite level, including fatty acid profiles in HM of mothers delivering term over 6 months of lactation. Furthermore, we have also recently reported on the HM microbiome in this cohort of lactating women over the course of lactation from birth to 6 months (75).

Though some variations in metabolite levels observed in this study have been observed in previous studies as discussed, we here add to the knowledge on HM metabolome as some changes in metabolite levels observed in this study are predominantly found in mature milk after 1 to 3 months of lactation. However, whereas for other metabolites, we demonstrate that although it has been previously observed in several studies that particularly lactose, glutamate, alanine, and 2′FL change in levels over lactation, we here observe that the changes are significant within the first month of lactation from transitional to mature milk, after which the levels are stable. As previously mentioned, glutamate and glutamine have previously been observed to increase in levels over 1 month of lactation in another metabolomic study, though we here show that glutamine continuously increase in levels in late mature milk, whereas levels of glutamate remain stable after 1 month of lactation. Increased levels of glutamine and glutamate over lactation may provide nitrogen for protein synthesis and gut maturation coherently with increased nutritional demand of the infant. Moreover, TCA cycle intermediates, including citric acid, have been observed to correlate with early lactation, and studies have observed distinct tendencies in the variation in levels over lactation, with this study adding to the knowledge on metabolites related to energy metabolism. Increased levels of TCA cycle intermediates early in lactation could reflect a shift in energy metabolism in the mammary gland throughout lactation, potentially to accommodate a changing need in the infant or be related to mother's milk production. Increasing levels of butyrate, caprate, caprylate, and valerate and variation in levels of fatty acid derivatives, including choline, O-phosphocholine, and ethanolamine, could reflect shifts maternal metabolism or related to the composition of the milk fat globule membrane. Moreover, whereas the concentration of most HMOs declines over lactation, that of 3-FL increases continuously, offering a prebiotic component to the gut environment of the infant. Combined, these results indicate that the composition of HM metabolome continuously changes throughout lactation to potentially accommodate the growing infant's needs and/or reflect shifts in mammary gland metabolism. However, the biological implications of these variations are not investigated in this study, and interpretations of the relations are therefore purely speculative. Nevertheless, these changes influence both nutritional and non-nutritional components provided for the infant. Thus, the results of this study support further in-depth research into the dynamic nature of late mature HM to investigate the impact on infant growth and development.

CONCLUSION

This study demonstrates how HM from mothers delivering at term changes over a period beyond the state of maturity from early lactation to late lactation (weeks 1–25 postpartum). Besides from the variation in agreement with similar studies in terms of levels of lactose, glutamine, glutamate, butyrate, caprate, caprylate, citric acid, and HMOs, we additionally found O-acetylcarnitine, gluconate, ethanolamine, and cis-aconitate to be correlated positively with early lactation, and 2-aminobutyrate, and valerate to be correlated positively with later lactation. Some metabolites change in concentration mainly throughout the first month after birth and afterward remain stable whereas, for others, the concentration is dynamic and continuously changes throughout lactation. This dynamic change in concentration is particularly evident for 6-SL, gluconate, glutamine, LNT, LNDFH I, and O-acetylcarnitine.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of Cork Teaching Hospitals, Cork, Ireland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ALK, EL, CS, and CAR: conceptualization. EL and UKS: methodology. KOP: formal analysis, investigation, writing—original draft preparation, and visualization. ALK and UKS: resources, project administration, and funding acquisition. CAR, ALK, and UKS: data curation. ALK, FM, UKS, and JFY: writing—review and editing. UKS and JFY: supervision. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.917659/full#supplementary-material

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REVIEW
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Effects of Antioxidants in Human Milk on Bronchopulmonary Dysplasia Prevention and Treatment: A Review

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Bronchopulmonary dysplasia (BPD) is a severe chronic lung illness that affects neonates, particularly premature infants. It has far-reaching consequences for infant health and their families due to intractable short- and long-term repercussions. Premature infant survival and long-term quality of life are severely harmed by BPD, which is characterized by alveolarization arrest and hypoplasia of pulmonary microvascular cells. BPD can be caused by various factors, with oxidative stress (OS) being the most common. Premature infants frequently require breathing support, which results in a hyperoxic environment in the developing lung and obstructs lung growth. OS can damage the lungs of infants by inducing cell death, inhibiting alveolarization, inducing inflammation, and impairing pulmonary angiogenesis. Therefore, antioxidant therapy for BPD relieves OS and lung injury in preterm newborns. Many antioxidants have been found in human milk, including superoxide dismutase, glutathione peroxidase, glutathione, vitamins, melatonin, short-chain fatty acids, and phytochemicals. Human milk oligosaccharides, milk fat globule membrane, and lactoferrin, all unique to human milk, also have antioxidant properties. Hence, human milk may help prevent OS injury and improve BPD prognosis in premature infants. In this review, we explored the role of OS in the pathophysiology of BPD and related signaling pathways. Furthermore, we examined antioxidants in human milk and how they could play a role in BPD to understand whether human milk could prevent and treat BPD.

Keywords: bronchopulmonary dysplasia, premature infants, oxidative stress, human milk, antioxidants

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INTRODUCTION

BPD is the most prevalent complication in preterm infants. In 1967, Northway et al. first defined BPD as a persistent lung injury caused by a high concentration of oxygen and high pressures during mechanical ventilation. "Classical BPD" or "old BPD" is diagnosed in "those newborns who require supplementary oxygen on post-natal day 28" and those who display radiographic abnormalities in the chest, such as emphysema, atelectasis, and vesical shadows (1). The survival rate of premature infants has improved dramatically in the decades since BPD was identified, owing to a substantial increase in the quality of nursing for premature infants, such as the development of non-invasive ventilation techniques and prenatal use of corticosteroids for increased alveolar surface area. However, the incidence of BPD remains relatively high. Moreover, the advancement of these tools has increased our understanding of BPD; the definition of "old BPD" is no longer appropriate for current diagnostic criteria.

In 2001, Jobe et al. updated the diagnostic criteria and definition of BPD (NIH), also known as "New BPD": infants with oxygen dependence (inhaled oxygen concentration [FiO_2] >21%) for >28 days should be diagnosed with BPD, and classified as mild, moderate, severe BPD based on aerobic conditions at 36 week post-menstrual age (PMA). The new BPD highlights the link between alveolar dysplasia and lung injury in preterm infants (2, 3). In 2018, the National Institute of Child Health and Human Development modified its rules, removing the need for oxygen dependence for >28 days (4). In 2019, Jensen et al. proposed that the degree of oxygen reliance should not be considered when diagnosing BPD; instead, the mode of assisted breathing should be considered (5). For clinicians, an accurate diagnosis of BPD would allow the development of individualized respiratory support measures and medication therapy, which are advantageous for the rehabilitation and prognosis of patients with BPD.

Between 2010 and 2019, the incidence of BPD among extremely premature newborns in China was >74%; the younger the PMA, the higher the incidence of BPD (6). However, the treatment of BPD is controversial. A worse prognosis usually accompanies a higher disease severity. Currently, the most common treatment options for BPD are delivery room intervention (7, 8), invasive mechanical ventilation (9, 10), steroids (11), diuretics (12), bronchodilators (13), and caffeine (14, 15). Furthermore, dietary support, infection management, and vasodilators are beneficial as therapy for BPD (16). The importance of OS in the etiology of BPD has been increasingly recognized, and the use of antioxidants in the treatment of BPD is becoming a popular research topic. Reducing the damage caused by oxidative free radicals to the lungs of preterm infants by maintaining the balance between oxidation and antioxidant systems is expected to provide a new method for preventing and treating BPD (17, 18).

Human milk is the most comprehensive and natural nourishment for infant growth. It contains all the nutrients and biologically active components that newborns require to meet their developmental demands and boost their immunity. Breast milk contains various antioxidants, such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), vitamins, phytochemicals, melatonin, probiotics, short-chain fatty acids (SCFAs), and unique human milk oligosaccharides(HMOs), milk fat globule membrane(MFGM), and lactoferrin, which provide a solid antioxidant capacity for newborns. A meta-analysis on breastfeeding and BPD found that the incidence of BPD decreased when premature newborns were exclusively breastfed compared to when they were formula fed (19). In a cohort study, the amount of human milk consumed by premature newborns was inversely associated with the incidence of BPD, with a daily intake of 7 ml/(kg·d) showing a BPD prevention effect (20). Aloka et al. found that providing sufficient nutritional care to premature children with low birth weight is significant for preventing BPD. For every 10% increase in breastfeeding in preterm children from birth to 36 weeks PMA, the risk of BPD is lowered by 9.5% (21). Breastfeeding also reduces the risk of newborn problems in the neonatal intensive care unit (NICU) (22). Yan et al. discovered that a daily intake of 50 ml/(kg·d) of human milk from the mother during the first 4 weeks after delivery can minimize the incidence of BPD in infants (23). According to these studies, human milk appears to serve a beneficial function in the prevention and treatment of BPD. Given the presence of different antioxidants in breast milk, we believe that this is the primary mechanism through which human milk contributes to decreased BPD. Therefore, this review examined the critical role of OS in BPD and the impact of antioxidants in human milk on the prevention and treatment of BPD. We have also discussed future considerations for clinical BPD treatment.

Abbreviations: BPD, Bronchopulmonary dysplasia; OS, Oxidative stress; NICHD, The National Institute of Child Health and Human Development; ES, Endoplasmic reticulum stress; UPR, Unfolded protein response; VEGF, Vascular endothelial growth factor; MSC, Mesenchymal stem cells; Nrf2, Nuclear erythroid-E2-related factor 2; ARE, Antioxidant reaction elements; HO-1, Heme oxygenase-1; SIRT1, Silent mating-type information regulation 2 homolog 1; NAD, Nicotinamide adenosine dinucleotide; SENP1, Small ubiquitin-like modifier (SUMO)-specific protease 1; PBMC, Peripheral blood mononuclear cells; PPARγ, Peroxy-proliferator-activated receptor γ; AT2, Primary alveolar type 2 cells; TXN, Thioredoxin; TXNRD, Thioredoxin reductase; ATG, Aurothioglucose; NF-κB, Nuclear factor κB; Tet, Tetralin; ISAPP, The International Scientific Association for Probiotics and Prebiotics; 2′-FL, 2′-fucosyllactose; LNnT, Lacto-N-neotetraose; 3′-GL, 3′-galactosyl lactose; HMO, Human milk oligosaccharides; DPPH, 2, 2-diphenyl-1-hydrazide; SCFA, Short chain fatty acids; SOD, Superoxide dismutase; GSH-Px, Glutathione peroxidase; NQO1, NAD (P)H:quinone oxidoreductase.

Overview

OS is a condition in which there is an imbalance between reactive oxygen species (ROS) production and antioxidant capability. Under normal conditions, electrons created by the aerobic metabolism of cells are readily accepted by oxygen, resulting in ROS production. Excessive levels of ROS can harm cell structure, proteins, lipids, and nucleic acids, leading to cell death (24). The factors that influence the results of cell injury include interaction between specific molecules, the body's internal environment, cell location, time, and concentration (25). Premature newborns are more likely to develop BPD, especially those who require mechanical ventilation (**Figure 1**). Owing to respiratory system immaturity and pulmonary hypoplasia, premature infants require long-term oxygen therapy, which can expose them to a greater risk of illness and inflammation than term infants. They are also more likely to accumulate ROS due to high concentration oxygen dependence. Additionally, the endogenous antioxidant enzyme systems are defective in premature infants. SOD, catalase (CAT), and GPX concentrations at birth are substantially lower in premature newborns than in full-term infants; therefore, they are unable to effectively eliminate excess ROS, resulting in ROS accumulation and OS (26).

OS and Lung Injury

Lung Development Injury

OS can cause several lung injuries. Long-term hyperoxia in the lungs of premature newborns causes lung damage directly through ROS production (27). Alveolar epithelium type II cells (AEC II) are the most critical cells in the lung development stage and act as lung tissue stem cells. AEC II synthesizes and secretes

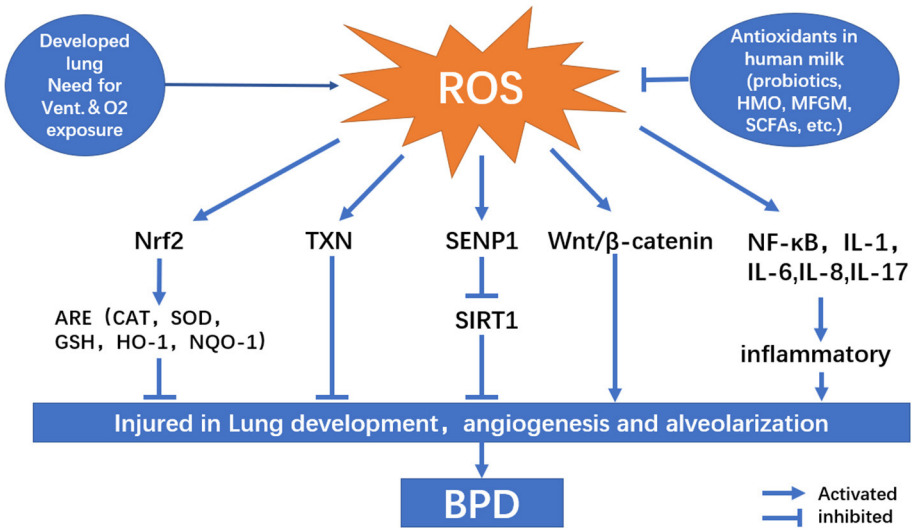

FIGURE 1 | Signaling pathway related to oxidative stress on bronchopulmonary dysplasia pathogenesis.

pulmonary surfactant, regulates lung tissue growth, repairs wounds, and adjusts alveolar moisture to maintain homeostasis. AEC II can develop into alveolar epithelial type I cells to repair the alveolar wall and preserve alveolar function when the alveolar–capillary barrier is damaged. A high concentration of oxygen can induce the apoptosis of AEC II and inhibit their proliferation. Injury to airway epithelial cells causes pulmonary tracheal remodeling and vascular remodeling, both of which are important in BPD etiology (28, 29). Airway remodeling occurs throughout infant lung development *via* several pathogenic processes involving airway epithelial cells, inflammatory cells, smooth muscle cells, and extracellular matrix. The leading causes of airway remodeling are aberrant airway healing and airway epithelial damage caused by decreased AEC II levels (30).

In addition, endoplasmic reticulum stress (ES) in the lungs can be exacerbated by OS in hyperoxic environments (31). Hyperoxia exposure can harm mitochondrial activity in the lungs, resulting in ES and alveolar injury, activating the pulmonary unfolded protein response (UPR), and increasing UPR downstream effectors, in turn resulting in alveolar growth disorder (32).

Arrested Alveolarization

Alveolarization (production of alveolar gas exchange units) can be impeded in newborns with BPD, although the mechanisms of alveolarization are unknown (33). Pulmonary alveoli are important gas exchange units, and alveolarization is a late goal in lung development. The terminal airspaces formed during early lung development are divided by secondary septation, resulting in an increasing number of smaller alveoli, thus improving gas exchangeability in the lungs. However, premature newborns with developed lungs are exposed to hyperoxia-obstructed gas exchange with continuous alternation of the bronchial, resulting in lung function disorder. In a previous study, neonatal mice treated with high oxygen (85% O_2) showed delayed alveolar growth, loss of bronchoalveolar attachment, increased lung compliance, and increased medial arteriole wall thickness, which persisted into adulthood. Alveolar defects have also been linked to reduced functional gas exchange (34).

Supplemental oxygen levels influence the severity of alterations in airway anatomy and function in neonates. Mild (40% O_2) and moderate (60% O_2) oxygen exposure can cause alterations in airway function and hyperresponsiveness in animals, but the effects are minor. The degree of OS is directly associated with interrupted alveolarization due to severely high oxygen (80% O_2) levels, which cause simplified alveolar, airway embolization, and elastin redistribution (35). Even a short period of hyperoxia lung injury (limited to cystic lung development) is sufficient to damage alveolar growth and reduce gas exchange function; irrespective of whether confined to the cystic period or chronic fetal hyperoxia, it will lead to reduced alveolar surface area volume and alveolar interval (36). Hyperoxia obstructs alveolarization, which is associated with mitochondrial malfunction. Both hyperoxia and direct suppression of mitochondrial oxidative phosphorylation can halt alveolar development. Long-term mechanical breathing inhibits the formation of pulmonary mitochondria, and this inhibition of oxidative phosphorylation persists throughout alveolar evolution, resulting in neonatal alveolar development delays (37, 38).

Pulmonary Vascular Injury

OS also impairs pulmonary angiogenesis. Pulmonary vascular abnormalities are another critical factor in BPD development; several growth factors play a role in pulmonary angiogenesis. The vascular endothelial growth factor (VEGF) can promote vascular endothelial cell proliferation and migration and reshaping of endothelial cells (39). VEGF also plays a crucial role in lung development by maintaining the typical structure and function of the alveoli during normal blood vessel development (40).

OS caused by ROS accumulation has been confirmed to decrease VEGF expression in mouse models, and abnormal VEGF signal transduction can impair angiogenesis and reduce alveolarization, resulting in the arrest of pulmonary vascular development and leading to experimental BPD (41). Studies have shown that both VEGF expression and lung capillary density are significantly decreased in patients with BPD and animal models and that increased VEGF expression can inhibit alveolar destruction induced by high oxygen levels (42). The combination of mesenchymal stem cells (MSCs) and erythropoietin has been shown to enhance lung protection in newborn mice under high-oxygen conditions. Increased expression of VEGF was found to alleviate lung injury in newborn BPD mice by indirectly promoting angiogenesis (43). Wallace et al., using anti-sF1t-monoclonal antibody therapy found that sF1t, an endogenous antagonist of VEGF, could prevent structural abnormalities in lung development and pulmonary hypertension in infancy and effectively preserve lung structure and function (44). Thus, early endothelial cell injury damages pulmonary blood vessels and alveolar formation. In addition, downregulation of VEGF expression further disrupts lung epithelial and mesenchymal development. Premature infants, born with developed lungs and long-term exposure to high oxygen demand, develop ROS accumulation, resulting in OS and reduced VEGF expression. Under hyperoxia, reduced levels of VEGF cannot effectively induce pulmonary angiogenesis and repair, and pulmonary angiogenesis ceases during germination. Insufficient angiogenesis leads to pulmonary vascular dysplasia and prevents alveolarization, eventually leading to BPD (45).

OS-Related Signaling Pathways

Nrf2–Keap1–ARE

Nuclear erythroid-E2-related factor 2 (Nrf2) is a major transcription factor involved in the antioxidant system that regulates OS. Nrf2 interacts with Keap1 in the cytoplasm and is destroyed by ubiquitination under normal conditions. The accumulation of ROS prevents Nrf2 from being ubiquitinated by modifying the cysteine in Keap1, allowing Nrf2 to be expressed stably in the nucleus and activating the antioxidant reaction elements (ARE), which include the expression of a series of antioxidant genes such as *GSH*, *GCL*, *HMOX*, and *GSL* (46). The oxidative equilibrium maintained by Nrf2 is especially crucial to the respiratory tract after long-term exposure to hyperoxia in the lungs of preterm newborns. Lack of Nrf2 can increase the sensitivity and severity of different respiratory conditions such as BPD, adult respiratory distress syndrome, chronic obstructive pulmonary disease, asthma, and lung cancer, whereas activation of Nrf2 protects against various respiratory diseases (47). The Nrf2-Keap1-ARE pathway is one of the most crucial protective mechanisms against BPD, probiotics, SCFAs, lactoferrin, arginine can activate this pathway and show their antioxidative effects. The pulmonary tissue structure of mice exposed to hyperoxia becomes disordered, the alveolar wall begins to weaken as the hyperoxia exposure period increases, and the concentration of antioxidative stress-related enzymes (CAT, SOD, and GSH-Px) decreases under hypoxia condition. The Nrf2 content is significantly higher in hyperoxic mice than in normal oxygen-treated mice. In contrast, the level of Keap1 is lower, indicating that the body can upregulate Nrf2 expression by inhibiting Keap1 expression in hyperoxic mice; subsequently, Nrf2 enters the nucleus to activate ARE and improve antioxidant capacity (48).

Targeting Keap1 knockdown to increase endogenous Nrf2 expression can be an approach for preventing low alveolarization in preterm infants. Keap1 knockdown has been shown to improve lung cell proliferation after birth in neonatal mouse models, accompanied by an increase in the expression level of the antioxidant gene (nuclear GSH). These findings indicate that Nrf2 plays an essential role in the development of neonatal lung tissue (49). The Nrf2 pathway has several effects on oxidation regulation, such as increasing heme oxygenase-1 (HO-1) expression to improve antioxidant and anti-inflammatory ability. Its protection against development of BPD is mainly dependent on the enzymatic products carbon monoxide, bilirubin, and iron, which prevent alveolar simplification, improve vascular remodeling, and avoid lipid peroxidation (50). In addition, Nrf2 can activate NAD (P)H:quinone oxidoreductase 1(NQO1) to mitigate lung damage by inhibiting cell apoptosis in hyperoxia (51).

SENP1–SIRT1

Silent mating-type information regulation 2 homolog 1(SIRT1) is a deacetylase activated by nicotinamide adenosine dinucleotide (NAD+). It regulates gene expression by removing acetyl groups from proteins involved in processes such as apoptosis, inflammation, aging, and OS. Lack of SIRT1 can lead to a significant increase in ROS levels and inflammatory response, increasing NAD+ can activate SIRT1, such as resveratrol and some probiotics in breast milk. Small ubiquitin-like modifier (SUMO)-specific protease 1 (SENP1) regulates SIRT1 distribution in OS by participating in substrate de-SUMO modification (small ubiquitination related modification). SIRT1 undergoes SUMOylation, an essential post-translational modification. After SUMOylation, SIRT1 deacetylation is strengthened, and SIRT1 in the nucleus can suppress apoptosis by deacetylating p53. Through de-SUMO modification of SIRT1, SENP1 can reduce SIRT1 deacetylation. SIRT1 protein expression is considerably decreased in neonates with BPD, and the interaction between SUMO1 and SUMO2/3 is significantly weakened. These findings imply that a reduction in SIRT1 SUMOylation may play a role in the development of BPD (52–54).

Recent studies have shown that SIRT1 is linked to the pathogenesis of BPD (52). In peripheral blood mononuclear cells (PBMCs) of neonates with BPD, elevated ROS levels and upregulated SENP1 expression were found. Moreover, SIRT1 showed substantial spatial variations, with decreased expression in the nucleus and increased expression in the cytoplasm. SENP1 silencing can alleviate hyperoxic injury in the hyperoxic alveolar epithelial cell injury model, suggesting that SENP1 can inhibit SIRT1 deacetylation activity by regulating SIRT1 expression and distribution in the nucleus, promoting cell apoptosis, and playing an essential role in hyperoxia-induced lung injury (55). In newborns with BPD, nuclear SIRT1 expression in PBMCs

collected by tracheal aspiration is lower and nuclear SIRT1 localization is substantially lower than those in normal neonates (56). The level of ROS and pace of SIRT1 translocation in premature newborns receiving assisted oxygen are strongly oxygen concentration dependence and are associated with a reduction in SIRT1 expression, suggesting that OS can change the activity and distribution of SIRT1. Resveratrol inhibits ROS formation under hyperoxia exposure by blocking the SIRT1 nuclear plasma shuttle and increasing SIRT1 expression (57). Furthermore, through the SIRT1 pathway, budesonide and porcine lung phospholipid injection can exert a protective effect on premature BPD (58).

Wnt/β-Catenin

The Wnt/β-catenin signaling cascade is critical for lung formation during early stages. Wnt signaling occurs at 7 weeks of pregnancy, peaks at 17 weeks, and subsequently declines at 21 weeks (59). According to Zhang et al., Wnt transcription factors are found in low amounts in the respiratory epithelium and mesenchymal lining of the lungs. It plays a vital role in regulating lung development. In neonatal rat lung injury caused by hyperoxia, abnormal activation of Wnt/β-catenin and decreased expression of peroxisome proliferator-activated receptor γ were observed. These changes were linked to reduction in alveolar septal thickness, radial alveolar count, and alveolar augmentation after hyperoxia exposure (60). Alapati et al. found that inhibiting β-catenin expression increased alveolar remodeling and reduced the incidence of pulmonary vascular remodeling and pulmonary hypertension in newborn rats, suggesting the involvement of Wnt/β-catenin pathway in hyperoxia-induced alveolar damage (61).

The expression time and regulation of Wnt signaling during lung development are minimal, and the activation of an aberrant Wnt gene can hinder normal lung development. The development of BPD is complicated by exposure of the lung to high oxygen levels during the cystic stage (62). Jennifer et al. discovered that at the cystic stage, increased oxygen exposure causes aberrant activation of mesenchymal Wnt5A. Furthermore, a three-dimensional organotypic coculture system has revealed that Wnt/β-catenin signal transduction occurs in AEC II and fibroblasts and that targeted suppression of Wnt5A can alleviate hyperoxia-induced alveolar constriction. There are several approaches to ameliorate hyperoxia-induced alveolar epithelial cell injury (YAP and ETS1) *via* the Wnt/β-catenin signaling pathway. However, given that the Wnt/β-catenin signaling pathway is essential for normal lung development, targeted therapy for this pathway is controversial. To ensure lung development and injury repair in premature infants, it is essential to accurately match the treatment objectives to restore the balance of the lung development signaling pathway (63, 64).

TXN System

Thioredoxin (TXN) is a small-molecule protein that functions as a hydrogen carrier in cells. Thioredoxin reductase (TXNRD) is a dimer released with a NAPDH dependent FAD domain. The TXN system, which includes TXN, TXNRD, and NAPDH, regulates REDOX equilibrium, cell proliferation, and cell death. It is also involved in embryonic development and immunological metabolism (65). TXN and TXNRD are primarily expressed in pulmonary epithelial cells, alveolar macrophages, and bronchial chondrocytes in neonates, and they function as antioxidants (66). Recent studies have linked TXN system damage with hyperoxic injury and abnormal lung development, noting that the TXN system not only has a simple ROS detoxification function but also acts as a REDOX sensor when oxygen concentration changes, assisting newborns to complete the oxygen concentration conversion process from intrauterine relative hypoxia to hyperoxia exposure (67).

The TXN system efficiently prevents oxidative damage and contributes to REDOX-sensitive lung growth signals. TXN1 siRNA knockdown reduces cell viability after hyperoxia therapy, whereas TXN1 overexpression increases cell survival (68). To minimize lung injury in the BPD model, the antioxidant capacity of the TXN system is dependent on the activation of Nrf2 and an increase in lung epithelial HO-1 expression (69). According to Zhang et al., TXN1 overexpression reduced apoptosis and increased MSC proliferation in lung MSCs transplanted into recipients, SOD levels, and GPX levels (70). TXN1 is a new target for BPD treatment in neonatal lung disorders as it is resistant to hyperoxic lung injury. Increased expression levels of TXN1, TXN2, and TXNRD have been reported in hyperoxia. In GSH reductase-deficient mice, the TXN system showed an apparent antioxidant compensatory effect (71). Wall et al. found that using aurothioglucose (ATG) as a TXNRD1 inhibitor increased the total GSH content and GPX activity of ATG-treated mice under hyperoxia, suggesting that ATG could improve GSH-dependent antioxidant capacity (72). TXNRD inhibitors also have favorable effects on lung development. RNA sequencing in the lungs of mice exposed to >95% oxygen for the first 72 h of life revealed that ATG selectively controlled genes were primarily related to angiogenesis and vascular development and that ATG use increased pulmonary vascular density in mouse models (73).

NF-κB

OS stimulates nuclear factor κB (NF-κB), which regulates inflammatory factor synthesis and activation, nuclear protein translocation, and apoptosis (74). NF-κB is a significant regulator of the incidence, progression, and clearance of inflammation and plays a prominent role in lung inflammation. Although phosphorylation of IκBα (inhibitor of NF-κB α) and nuclear accumulation of NF-κB P65 have been linked to the severity of BPD in recent studies, the role of NF-κB in BPD is debated (75). In breast milk, melatonin, HMOs, and lactoferrin can regulate oxidative balance through NF-κB. OS simultaneously triggers the body's inflammatory response. When exposed to hyperoxia, monocytes, macrophages, lymphocytes, epithelial cells, endothelial cells, and the matrix produce many chemokines, resulting in an influx of inflammatory cells. These are the most critical factors in the mechanism of hyperoxia-induced lung injury. Late chronic inflammatory injury is an essential factor that leads to abnormal lung development (76). OS stimulates several pro-inflammatory factors and mediators, such as IL-1, IL-6, IL-8, IL-17, IL-24, MCP-1, and TNF-α, resulting in an inflammatory cascade (77–81). Inflammation levels in neonates

with BPD continue to rise after birth, progressively diminishing after 2 weeks. This trend is particularly pronounced in males and infants with very low birth weight, implying that more targeted treatment interventions should be implemented early in BPD (82).

Previous research has shown that persistent hyperoxia-induced NF-κB activation helps survival and lung growth in neonates. NF-κB activation contributes to the activation of cell protection target genes such as VEGF receptor 2. Inhibition of NF-κB expression in the developing lung lowers vascular proliferation and alveolar–capillary density. It produces reduced alveolarization similar to BPD, indicating that NF-κB activation can be a therapeutic intervention to prevent BPD (83, 84). However, recent studies have suggested that inhibition of NF-κB can reduce hyperoxia-induced newborn lung injury. Tetralin (Tet) enhances antioxidant levels and lowers inflammatory factor levels in hyperoxia-induced lung function decline in rats. Jiao et al. have reported that Tet achieves this by suppressing NF-κB (85). Li et al. used a recombinant human elastase inhibitor, elafin, to inhibit the NF-κB pathway in hyperoxia-exposed mice. They found that elafin reduced apoptosis, inhibited inflammatory cytokines, improved nuclear accumulation of NF-κB P65, and improved alveolarization (86). According to Chen et al., caffeine can also reduce nuclear apoptosis and inflammatory lung injury in lung tissue, lower OS levels, enhance alveolar growth, and protect lung development from oxidative damage in hyperoxia (87). Excessive activation or inhibition of the NF-κB pathway is detrimental to the normal development of the lungs, and direct targeting of the NF-κB pathway may further lead to pulmonary dysplasia. New methods for the prevention and treatment of BPD include targeting NF-κB signaling and suppressing dangerous downstream signals of the NF-κB pathway.

Therefore, OS plays a significant role in the etiology of BPD. Antioxidant treatment can lower infant lung OS, using that antioxidant treatment is an essential study direction for BPD. However, as the free radicals produced by BPD are diverse, the clinical effect of a single antioxidant treatment for BPD does not achieve the desired effect. Therefore, applying various antioxidants in breast milk may positively affect the prevention and treatment of BPD.

ANTIOXIDANTS IN BREAST MILK

Human milk is the safest and most natural nourishment for infants (88). It contains all the calories, proteins, and lipids that newborns require for growth and development and is also high in antioxidants (**Table 1**). Antioxidants are thought to exist in a range of active compounds. They can remove ROS or reactive nitrogen species directly, change the activity of enzyme boosting and antioxidant enzymes, or regulate REDOX signaling pathways to achieve their antioxidant actions. The overall antioxidant capacity of breastmilk is an important defense mechanism for preventing illnesses that affect newborns (**Table 2**). Breastfeeding has been shown to minimize the incidence of BPD in premature newborns in clinical practice.

Probiotics

Probiotics are microbes that are beneficial to human health. Human milk contains a complex microbial community, providing probiotics and vital energy for neonates. The International Scientific Association for Probiotics and Prebiotics defined probiotic calibration in 2013 as "providing a health benefit to the host when administered in sufficient numbers of living microorganisms," which includes microorganisms that have been shown to have a health benefit in controlled trials and new symbiotic strains from human samples (127). *Lactobacillus* and *Bifidobacterium* are the most prevalent probiotics found in human milk, which can dwell in the intestinal system of neonates along with other probiotics (128, 129). Chanettee et al. identified two *Lactobacillus* species, *L. plantarum* and *L. pentosus*, in human milk (130). Breast milk is the first source of intestinal bifidobacteria in newborns, and they dominate the intestinal flora of breastfed infants (131). The antioxidant capacity of probiotics can be observed in various ways. Probiotics can aid in the chelation of metal ions and removal of reactive free radicals. Their metal chelation action is strain specific. Moreover, probiotics can release antioxidant enzymes and active compounds, such as SOD, CAT, NADH oxidase, TXN, and GSH, which are abundant in *Lactobacillus* and *Bifidobacterium*, indicating that probiotics have a substantial antioxidant activity (132, 133). Furthermore, probiotics modulate oxidative equilibrium by modulating antioxidant signaling pathways, with antioxidant effects exerted *via* the Nrf2, SIRT1, MAPK, and PKC pathways, demonstrating strain specificity (134). Finally, by strengthening the integrity of the intestinal barrier, initiating the immune response, and avoiding inflammation and OS, probiotics and their metabolites can prevent additional microbial metabolites and endotoxins from entering the circulation (135).

Probiotics can lower the incidence of BPD in premature children with a PMA of <32 weeks, demonstrating the potential of probiotics in the treatment of BPD (136). Probiotics can enhance the recovery of the gut-lung axis, anti-inflammation, and anti-infection by modulating the balance of gut microbiota and their remarkable antioxidative powers. However, probiotics can also have adverse effects on the human body. Thus, the safety of probiotics in the human body should be carefully examined before using them to treat infant with BPD, and the species, dose, and frequency of administration should be carefully chosen (137, 138). Formula is an excellent substitute for human milk when new mothers cannot breastfeed owing to physical limitations. Formula adds a range of nutrients to approximate human milk constituents while meeting the nutritional needs of neonates. In addition to specific protein components, fatty acids, carbohydrates, probiotics, and prebiotics are added to formula to control the microbiota of infants (139).

HMOs

HMOs are the third most abundant solids in human milk, after lactose and fat. HMOs have potent anti-inflammatory, anti-infection, and prebiotic properties. They can also modulate the intestinal epithelial cell response and promote normal neonatal development through interactions with the gut flora. HMOs also play significant roles in adult brain development and

TABLE 1 | Antioxidants in human milk and differences between term (PMA > 37 weeks) and preterm (PMA < 37 weeks) newborn mothers' milk.

Substances and lactation stage		Value and unit in term infant HM		Value and unit in preterm infant HM		References
Total HMO						
Colostrum[a]	Mean ± SD[d]	12.5 ± 7.2[d]	g/L	/		(89)
		9.6 ± 6.1		/		(90)
		22.4 ± 4.6		/		(91)
		13.0 ± 3.9		/		(92)
Transitional milk[b]	Mean ± SD	11.0 ± 6.0	g/L	/		(89)
		8.4 ± 5.0		/		(90)
		18.9 ± 3.9		/		(91)
		10.7 ± 2.1		/		(92)
Mature milk[c]	Mean ± SD	9.6 ± 5.1	g/L	/		(89)
		6.6 ± 4.4		/		(90)
		14.6 ± 4.3		/		(91)
		9.2 ± 2.0		/		(92)
2′FL						
Colostrum	Mean ± SD	3.57 ± 1.90	g/L	2.50 ± 1.63	g/L	(93)
Transitional milk	Mean ± SD	2.20 ± 1.43	g/L	1.67 ± 1.12	g/L	(93)
		1.00 ± 0.06		/		(94)
Mature milk	Mean ± SD	2.00 ± 1.25	g/L	1.65 ± 1.47	g/L	(93)
	Mean	2.74		2.77		(95)
	Mean ± SD	0.84 ± 0.57		/		(94)
LNnT						
Colostrum	Mean ± SD	0.33 ± 0.09	g/L	0.27 ± 0.12	g/L	(93)
Transitional milk	Mean ± SD	0.22 ± 1.00	g/L	0.22 ± 0.09	g/L	(93)
		1.22 ± 0.47		/		(94)
Mature milk	Mean ± SD	0.17 ± 0.08	g/L	0.18 ± 0.08	g/L	(93)
	Mean	0.74		0.66		(95)
	Mean ± SD	1.00 ± 0.42		/		(94)
3′–GL						
Colostrum	Mean ± SD	12.96 ± 6.37	mg/L	11.69 ± 8.00	mg/L	(93)
Transitional milk	Mean ± SD	7.13 ± 4.93	mg/L	6.21 ± 4.19	mg/L	(93)
Mature milk	Mean ± SD	5.54 ± 3.06	mg/L	5.58 ± 5.48	mg/L	(93)
Vitamin A						
Colostrum	Range	5–7	μmol/L	3	μmol/L	(96)
	Mean ± SD	146.9 ± 70.9	μg/100 mg	/	μmol/L	(97)
Transitional milk	Range	3–5	μmol/L	3.5	μmol/L	(96)
	Mean ± SD	81.8 ± 45.8	μg/100 mg	/		(97)
Mature milk	Range	1.4–2.6	μmol/L	2	μmol/L	(96)
	Mean ± SD	1.87 ± 0.81		1.38 ± 0.67		(98)
	Mean ± SD	1.76 ± 0.85		/		(99)
	Mean ± SD	59.5 ± 51.6	μg/100 mg	/		(97)
α tocopherol						
Colostrum	Mean ± SD	9.99 ± 1.51	mg/L	/	mg/L	(100)
		/		7.77 ± 2.88		(101)
	Mean ± SD	612.6 ± 412.3	μg/100 g	/		(97)
	Median (Q1,Q3)[e]	840.40 (671.18,1157.02)	μg/100 ml	/		(102)
Transitional milk	Mean ± SD	4.45 ± 0.95	mg/L	/	mg/L	(100)
		/		4.68 ± 2.94		(101)
	Mean ± SD	248.5 ± 218.5	μg/100 g	/		(97)
	Median (Q1,Q3)	418.68 (322.39,535.20)	μg/100 ml	/		(102)

(Continued)

TABLE 1 | Continued

Substances and lactation stage		Value and unit in term infant HM		Value and unit in preterm infant HM		References
Total HMO						
	Mean ± SD	2.92 ± 0.84	mg/L	/		(100)
	Median (Q1,Q3)	3.16 (2.29,4.16)		/		(103)
Mature milk	Mean ± SD	177.1 ± 109.0	μg/100 g	/		(97)
	Median (Q1,Q3)	290.6 (223.98,382.00)	μg/100 ml	/		(102)
γ tocopherol						
Colostrum	Mean ± SD	0.57 ± 0.21	mg/L	/		(100)
	Median (Q1,Q3)	110.07 (72.93,165.14)	μg/100 ml			(102)
Transitional milk	Mean ± SD	0.60 ± 0.21	mg/L	/		(100)
	Median (Q1,Q3)	76.78 (47.27,113.42)	μg/100 ml	/		(102)
Mature milk	Mean ± SD	0.30 ± 0.14	mg/L	/		(100)
	Median (Q1,Q3)	0.89 (0.58,1.27)		/		(103)
	Median (Q1,Q3)	57.28 (35.60,84.70)	μg/100 ml			(102)
SCFA						
Formate (C1:0)						
Mature milk	Mean (range)	43.7 (15.2–4,960.3)	μmol/L	/		(104)
Acetate (C2:0)						
Mature milk	Mean (range)	46.8 (13.5–4,307.7)	μmol/L	/		(104)
Butyrate (C4:0)						
Colostrum	Mean ± SD	0.06 ± 0.06	mg/g milk fat	0.12 ± 0.11	mg/g milk fat	(105)
Transitional milk	Mean ± SD	0.07 ± 0.05	mg/g milk fat	0.12 ± 0.09	mg/g milk fat	(105)
Mature milk	Mean ± SD	0.24 ± 0.20	mg/g milk fat	0.09 ± 0.07	mg/g milk fat	(105)
	Mean (range)	95.6 (4.8–409.5)	μmol/L	/		(104)
Lactoferrin						
Colostrum	Range	6–8	g/L	/	g/L	(106)
	Mean	3.16		/		(107)
	Mean ± SD	/		14.92 ± 7.96		(108)
	Mean	0.37		0.76		(109)
Transitional milk	Mean	1.73	g/L	10.73 ± 5.67	g/L	(107)
	Mean ± SD	/		0.58		(108)
	Mean	0.37				(109)
Mature milk	Range	1–2	g/L	/	g/L	(106)
	Mean	0.90		/		(107)
	Mean ± SD	/		10.34 ± 6.27		(108)
	Mean	0.30		0.39		(109)
	Mean ± SD	3.39 ± 1.43		/		(109)
Melatonin						
Colostrum (nighttime)	Mean	25.31	pg/ml	28.67	pg/ml	(110)
	Median (Q1,Q3)	36.9 (19.1,48.7)		/		(111)
Transitional milk (nighttime)	Mean	22.55	pg/ml	24.70	pg/ml	(110)
	Median (Q1,Q3)	30.8 (12.4,37.8)		/		(111)
				/		
	Median (Q1,Q3)	70.7 (26.3,129.7)				(112)
Mature milk (nighttime)	Mean	20.12	pg/ml	22.37	pg/ml	(110)
	Median (Q1,Q3)	32.6 (15.6–47.9)		/		(111)
	Median (Q1,Q3)	31.3 (23.2,51.1)		/		(112)
	Mean (range)	3.9 (0.8–36.2)		2.7 (0.1–30.5)		(113)

(Continued)

TABLE 1 | Continued

Substances and lactation stage		Value and unit in term infant HM		Value and unit in preterm infant HM		References
Total HMO						
Phytochemicals						
epicatechin						
Transitional milk	Mean (range)	90.5 (68.3–120.5)	nm/L	/		(114)
Mature milk		249.2 (63.7–828.5)		/		
Epicatechin gallate						
Transitional milk	Mean (range)	189.5 (55.7–609.3)	nm/L	/		(114)
Mature milk		236.6 (62.2–645.6)		/		
Epigallocatechin gallate						
Transitional milk	Mean (range)	1,118.8 (425.5–2,364.7)	nm/L	/		(114)
Mature milk		667.2 (215.1–1,683.8)		/		
Naringenin						
Transitional milk	Mean (range)	251.2 (82.9–542.6)	nm/L	/		(114)
Mature milk		210.4 (98.1–722.0)		/		
Kaempferol						
Transitional milk	Mean (range)	15.7 (7.8–34.0)	nm/L	/		(114)
Mature milk		23.1 (8.9–53.6)		/		
Hesperetin						
Transitional milk	Mean (range)	459.2 (107.1–1,272.8)	nm/L	/		(114)
Mature milk		393.6 (79.9–1,603.1)		/		
Quercetin						
Transitional milk	Mean (range)	48.1 (40.0–77.6)	nm/L	/		(114)
Mature milk		59.8 (33.1–108.6)		/		
α-carotene						
Transitional milk	Mean (range)	59.0 (12.0–220.6)	nm/L	/		(114)
Mature milk		19.2 (7.3–46.5)		/		
β-carotene						
Colostrum	Median (Q1,Q3)	11.14 (6.47,23.27)	μg/100 ml	/		(102)
Transitional milk	Mean (range)	164.3 (17.3–327.8)	nm/L	/		(114)
	Median (Q1,Q3)	3.13 (1.78,5.3)	μg/100 ml	/		(102)
Mature milk	Mean (range)	104.4 (10.7–377.4)	nm/L	/		(114)
	Median (Q1,Q3)	1.77 (1.03,3.07)	μg/100 ml	/		(102)
α-cryptoxanthin						
Transitional milk	Mean (range)	30.6 (11.9–72.6)	nm/L	/		(114)
Mature milk	Mean (range)	16.8 (2.2–50.6)	nm/L	/		(114)
β-cryptoxanthin						
Colostrum	Median (Q1,Q3)	3.90 (1.54,7.02)	μg/100 ml	/		(102)
	Median (Q1,Q3)	754.6 (429.6,1,486)	nm/L	406.7 (231.4,853.0)	nm/L	(115)
Transitional milk	Mean (range)	57.4 (9.4–175.4)	nm/L	/		(114)
	Median (Q1,Q3)	1.99 (1.31,3.56)	μg/100 ml	/		(102)
Mature milk	Mean (range)	27.5 (2.1–70.7)	nm/L	/	nm/L	(114)
	Median (Q1,Q3)	190.6 (87.4,353.9)		135.1 (53.0,224.6)		(115)
	Median (Q1,Q3)	0.82 (0.46,2.08)	μg/100 ml	/		(102)
Zeaxanthin						
Colostrum	Median (Q1,Q3)	2.15 (1.27,3.34)	μg/100 ml	/		(102)
	Median (Q1,Q3)	106.4 (73.7,141.4)	nm/L	63.2 (35.9,112.6)	nm/L	(115)

(Continued)

TABLE 1 | Continued

Substances and lactation stage		Value and unit in term infant HM		Value and unit in preterm infant HM		References
Total HMO						
Transitional milk	Mean (range)	46.3 (19.4–115.4)	nm/L	/		(114)
	Median (Q1,Q3)	2.21 (1.33,2.95)	μg/100 ml	/		(102)
Mature milk	Mean (range)	22.8 (11.9–52.6)	nm/L	/	nm/L	(114)
	Median (Q1,Q3)	59.9 (38.4,91.1)		46.6 (36.7,65.9)		(115)
	Median (Q1,Q3)	1.11 (0.7,1.93)	μg/100 ml	/		(102)
Lutein						
Colostrum	Median (Q1,Q3)	7.12 (5.13,13.03)	μg/100 ml	/		(102)
	Median (Q1,Q3)	486.3 (322.9,745.8)	nm/L	432.8 (231.6,667.9)		(115)
Transitional milk	Mean (range)	121.1 (58.1–412.9)	nm/L	/		(114)
	Median (Q1,Q3)	9.49 (6.77,13.1)	μg/100 ml			(102)
Mature milk	Mean (range)	61.9 (16.8–193.4)	nm/L	/	nm/L	(114)
	Median (Q1,Q3)	195.9 (150.3,270.8)		217.3 (170.0–283.0)		(115)
	Median (Q1,Q3)	4.57 (2.95,7.56)	μg/100 ml	/		(102)
Lycopene						
	Median (Q1,Q3)	11.88 (7.09,17.43)	μg/100ml	/		(102)
	Median (Q1,Q3)	1,065 (483.0,1846)	nm/L	669.9 (388.1,931.6)	nm/L	(115)
Transitional milk	Mean (range)	119.9 (30.5–317.5)	nm/L	/		(114)
	Median (Q1,Q3)	1.26 (0.96,2.52)	μg/100 ml	/		(102)
Mature milk	Mean (range)	58.0 (9.0–256.6)	nm/L	/	nm/L	(114)
	Median (Q1,Q3)	192.7 (118.8,221.2)		125.8 (89.4,173.6)		(115)
	Median (Q1,Q3)	0.58 (0.29,0.99)	μg/100 ml	/		(102)
GSH						
Transitional milk	Median (Q1,Q3)	20.2 (15.8,22.5)	Mg/ml	/		(112)
Mature milk		12.2 (10.5,15.8)		/		
SOD						
Colostrum	Mean ± SD	33 ± 15	units/ml	32 ± 20	units/ml	(116)
Transitional milk		43 ± 25		35 ± 16		
Mature milk		36 ± 11		37 ± 20		
Mature milk	Median (Q1,Q3)	202 (117–243)	ng/mol	245 (179–353)	ng/mol	(113)
CAT						
Transitional milk	Median (Q1,Q3)	261.5 (256.6,271.1)	units/ml	/		(112)
Mature milk		262.0 (253.5, 274.7)		/		
GPX						
Colostrum	Mean ± SD	9.0 ± 3.9	mU/mg	6.9 ± 1.6	mU/mg	(116)
Transitional milk		11.0 ± 4.3		10.0 ± 5.1		
Mature milk						
		10.1 ± 3.6		9.6 ± 2.5		
Mature milk	Median (Q1,Q3)	1,505 (799–2,269)	ng/mol	1,591 (1,174–2,208)	ng/mol	(113)
Iron						
Colostrum	Mean ± SD	0.11 ± 0.43	Mg/L	1.10 ± 0.34	Mg/L	(117)
Transitional milk		0.99 ± 0.31		0.99 ± 0.27		

(Continued)

TABLE 1 | Continued

Substances and lactation stage		Value and unit in term infant HM		Value and unit in preterm infant HM		References
Total HMO						
Mature milk		0.88 ± 0.28		0.90 ± 0.23		
Mature milk	Mean (range)	313.0 (24.2–2,157.1)	μg/L	380.0 (110.5–3,594.0)	μg/L	(118)
Copper						
Colostrum	Mean ± SD	0.72 ± 0.13	Mg/L	0.83 ± 0.21	Mg/L	(117)
Transitional milk		0.73 ± 0.21		0.78 ± 0.18		
Mature milk		0.58 ± 0.09		0.63 ± 0.14		
Mature milk	Mean (range)	288.8 (81.6–829.0)	μg/L	618.3 (95.4–954.5)	μg/L	(118)
Zinc						
Colostrum	Mean ± SD	5.35 ± 1.20	Mg/L	5.30 ± 1.45	Mg/L	(117)
Transitional milk		4.10 ± 0.65		4.75 ± 1.56		
Mature milk		2.60 ± 0.65		3.92 ± 1.10		
Mature milk	Mean (range)	1,434.3 (76.3–9,632.0)	μg/L	2,614.4 (422.8–17,727.2)	μg/L	(118)
Selenium						
Mature milk	Mean (range)	8.4 (2.5–38.1)	μg/L	12.6 (3.0–70.6)	μg/L	(118)
Arginine						
Colostrum	Mean	94.3	μmol/L	/		(119)
Transitional milk		35.6		/		
Mature milk		30.2		/		
Glutamine						
Colostrum	Mean	13.5	μmol/L	/		(119)
Transitional milk		92.6		/		
Mature milk		134.6		/		

Lactation Stage:
[a] *The earliest secreted milk after birth and in the first week postpartum (days 0–5).*
[b] *The milk is expressed in the first or second week (days 6–15).*
[c] *The milk secreted at the latest 4 weeks postpartum.*
[d] *Data are expressed as mean ± standard deviation (SD).*
[e] *Data are expressed as median (interquartile range).*

cognitive function. The HMO content in mature human milk is approximately 12–15 g/L, and it gradually declines from colostrum to mature milk (140). Studies have also demonstrated that mothers of highly malnourished newborns have lower levels of validated acidic HMOs and fucosylated neutral HMOs than those of normal newborns (141). HMOs in human milk can bind to lactose to form an indigestible trisaccharide or tetrasaccharide; sialic acid can be added to create sialyllactoses (3′-SL and 6′-SL) and fucose can be added to form fucosyllactoses (2′-FL and 3-FL) (142).

The most prevalent oligosaccharide in human milk is 2′-FL. It comprises 30% of HMO. Tu et al. demonstrated that 2′-FL could react with whey protein and remove 2, 2-diphenyl-1-hydrazide radicals, resulting in high antioxidant activity. Further, 2′-FL-fortified formula improves intestinal protection, is well accepted by newborns, and has absorption and excretion rates comparable to those of human milk (143). Lacto-N-neotetraose (LNnT) is a neutral HMO that is not fucosylated. Both 2′-FL and LNnT are new prebiotic additives in formula that make the composition of formula similar to that of human milk. They demonstrate clinical benefits in terms of intestinal probiotic protection, immunological modulation, and brain development and are a safe addition to infant formula (122, 144). Another HMO found in human milk and fermented formula is 3′-galactosyl lactose (3′-GL), which inhibits NF-κB inflammatory signaling and preserves the intestinal barrier. As 3′-GL is a by-product of fermented formula, its safety has been established over time (145).

Most HMOs can reach the intestine, operate as metabolic substrates for the intestinal flora, interact with the intestinal flora, and play a role in modifying the immune system and eliminating pathogens in newborns with low milk oligosaccharide absorption rates. HMOs also act as prebiotics, encouraging the development of newborn intestinal *Bifidobacterium* and enhancing SCFA synthesis (146). HMOs can also produce butyrate when they react with intestinal bacteria (147). Given the critical role of HMOs in the development and antioxidant capacity of the intestinal flora, more forward-looking, thorough clinical research is needed to evaluate the association between HMOs and BPD.

TABLE 2 | Antioxidants in human milk and their antioxidative functions to BPD.

Bioactive molecules	Antioxidative function	Clinical trials/formula	References
Probiotics			
Lactobacillus Bifidobacterium	Metal chelation; Secretion of antioxidant enzymes and active antioxidant substances; Enhance antioxidant signaling pathway; Reduce free radical production; Protect gut microbiome	Supplement of Lactobacillus paracasei strain F19 in formula is safe and well-tolerated for newborns and reduces occurrence of upper respiratory infection ($n = 200$); Probiotics can reduce the incidence of BPD in preterm infants under 32 weeks gestational age ($n = 318$)	(120, 121)
HMO			
2′FL; LNnT; 3′-GL	Reduce free radical production; Protect gut microbiome	Infant formula supplemented with 2′FL (1 g/L) and LNnT (0.5 g/L) is safe, well-tolerated, and reduces the incidence of bronchitis ($n = 88$)	(122)
MFGM	Regulate intestinal flora	Adding bovine MFGM to formula is safe before newborn is 2 years old ($n = 582$); MFGM added to formula is safe and well-tolerated in neonates with few adverse reactions ($n = 200$)	(120, 123)
SCFA	Regulate intestinal flora; Anti-inflammation	/	
Lactoferrin	Inhibit lipid peroxidation; Enhance antioxidant signaling pathway	Enteral lactoferrin supplementation at 100 mg/day did not affect BPD morbidity and mortality in preterm infants with GA≤32 weeks ($n = 2,182$).	(124)
Melatonin	Reduce free radical production; Promote production of antioxidant enzymes	Melatonin treatment reduce mortality and hospital stay of BPD in preterm infants ($n = 80$).	(125)
Vitamin			
Vitamin A Vitamin E	Reduce free radical production; Inhibit lipid peroxidation Reduce free radical production	Early oral administration of vitamin A (5,000 IU vitamin A/kg/ day) at 28 days postpartum in very low birth-weight infants reduced BPD morbidity and mortality ($n = 457$)	(126)
GSH	Reduce free radical production; Participate in Vitamin C and Vitamin E cycles	/	
Phytochemicals polyphenols carotenoids	Metal chelation; Reduce free radical production; Protect gut microbiome; Inhibition of oxidase; Converse to Vitamin A	/	
SOD	Catalyze transformation of superoxide anion free radicals into hydrogen peroxide	/	
CAT	Decomposition of hydrogen peroxide; Prevent accumulation of oxidative free radicals		
GPX	Decomposition of hydrogen peroxide; Avoid accumulation of oxidative free radicals		
Free amino acid	Synthesis NO; Promote GSH generation; Promote production of antioxidant enzymes	/	
Trace element	Catalyze REDOX reaction; Promote the antioxidant system	/	

MFGM

MFGM is a fat droplet that is essential for lipid transport in human milk. It comprises three layers—phospholipid (PL), sphingomyelin (SM), and various protein–membrane structures. PL and SM are derived from alveolar epithelial cells. PL comprises the main glycerol chain, whereas SM is composed of the main ceramide chain. MFGM promotes proper brain development by improving neonatal immunological function, controlling the intestinal flora, and boosting neonatal immune function (148, 149). MFGM can interact with intestinal probiotics, increase the adhesion of probiotic bacteria in the gut, and increase residence time in the gut (150). The addition of MFGM to formula can help minimize variations in the intestinal flora between breastfeeding and formula feeding (151). MFGM supplementation can assist in maintaining the integrity of intestinal epithelial cells and reducing inflammatory responses in mice with lipopolysaccharide-induced inflammation, implying that MFGM has anti-inflammatory properties (152). An increasing number of trials involving the addition of MFGM to formula have been conducted in recent years. Clinical studies have indicated that MFGM in formula is safe and well tolerated, with few side effects, similar to those observed in breastfeeding babies (120, 123). There are currently no recommended trials on MFGM supplementation in infants with BPD.

SCFAs

SCFAs, such as acetic acid, propionic acid, and butyric acid, are created by human gut flora metabolism and play a significant role in metabolism, the immune system, and anti-inflammation (153). The amount of SCFAs in human milk varies throughout the lactation period. Mature milk has the highest amount of SCFAs, which is four to seven times that in colostrum and transitional milk (105). SCFAs in human milk have been linked to newborn weight gain and obesity (154). SCFAs can enhance lung development and prevent lung disorders through the gut-lung axis. For example, butyrate, with a concentration of approximately 0.75 mM in human milk, protects the integrity of the intestinal barrier and significantly reduces mitochondrial damage in mouse models. OS is controlled by reducing hydrogen peroxide release and regulating the expression of related enzymes (155). Propionate plays a clear role in maintaining the pulmonary immune response; an increase in propionate in the intestinal flora has an anti-inflammatory effect (156). In a previous study, lung inflammation was improved, inflammatory factor expression was reduced, and intestinal microbiota was regulated in mice with BPD treated with acetate (157). In an Nrf2-dependent manner, sodium propionate can reduce lung inflammation and OS and facilitate alveolar simplification and aberrant angiogenesis generated by lipopolysaccharide in mice (158). These findings suggest that SCFAs are crucial for treating lung disorders. Pulmonary OS in newborns with BPD can encourage the production of inflammatory factors and trigger an inflammatory response, aggravating OS. SCFAs have been shown to reduce pulmonary inflammation, which is beneficial for treating BPD. Therefore, SCFAs are essential metabolites of the gut flora. Infants with BPD have a maladjusted gut flora, and human milk can deliver other SCFAs to neonates with BPD.

Lactoferrin

Lactoferrin is a transferrin found in human and other mammalian milks. Lactoferrin is the most abundant protein in colostrum and possesses antibacterial, antioxidant, antiviral, and anticancer activities (159). Lactoferrin protects the body from OS in two ways—[1] it reduces the Fenton reaction and ROS production by binding to iron and preventing lipid peroxidation (106) and [2] it activates the NF-κB/MAPK pathway to relieve cellular inflammation, maintain cell barrier integrity, reduce OS, activate Nrf2 expression, upregulate GSH activity, and reduce ROS and MDA production (160). Lactoferrin is well tolerated in very young newborns, is straightforward to administer, and serves as a reference for lactoferrin supplementation in clinical practice (161). Lactoferrin supplementation for very young newborns has been shown to minimize the risk of neonatal necrotizing enterocolitis and late sepsis (162, 163). However, some studies have refuted this finding, proposing that enteral supplementation of lactoferrin does not reduce the incidence of infection or other diseases in premature infants and does not reduce the incidence of BPD. The mechanism of lactoferrin in the prevention of BPD is unclear (124). In a study by Dobryk et al., enteral lactoferrin supplementation at 100 mg/day did not affect BPD morbidity and death in preterm infants at PMA $\leq$32 weeks. However, it may help achieve faster completion of enteral feeding and a shorter hospital stay in the most premature newborns (164).

Vitamins

Vitamin A

Vitamin A is a fat-soluble vitamin with biological activity and is a principal exogenous antioxidant. The lung is a critical target organ for vitamin A. Vitamin A can promote pulmonary vascular development, remove overabundant oxidative free radicals, and inhibit lipid peroxidation of the cell membrane, thereby reducing the effects of hyperoxia on lung development at various stages of alveolar development (165). Many studies have examined the critical role of vitamin A in the etiology of BPD. However, opinions on the prevention and treatment of BPD with early vitamin A supplementation are divided. In a clinical trial, early oral vitamin A (5,000 IU vitamin A/kg/day) minimized BPD morbidity and mortality in extremely low-birth-weight infants 28 days after delivery (126). In a study of very-low-birth-weight infants, Chabra et al. found that intramuscular injections of vitamin A can lower the incidence of BPD (166). High-quality reviews have demonstrated that early vitamin A supplementation for preterm newborns has good efficacy and safety, especially for extremely low-birth-weight infants. Vitamin A treatment can also reduce oxygen dependence for infants at a PMA of 36 weeks. However, as this supplementation effect has only been linked to a lower risk of BPD and not to lower risk of early death, more clinical research on amount and delivery of vitamin A supplementation is needed (167–170).

Although Abhijeet et al. demonstrated that enteral supplementation of water-soluble vitamin A improved plasma retinol levels in premature infants, they were unable to confirm a preventive or therapeutic effect on BPD, which might have been due to newborns' limited intestinal absorption capacity (171–173). The authors claimed that vitamin A supplementation might have a baseline—only vitamin A intake of 1,500 IU/kg/day was shown to reduce the incidence of BPD, regardless of the route of administration (enteral and parenteral) (174). In a phase III randomized controlled trial of 807 infants in 14 university hospitals across the United States, Matthew et al. found that vitamin A treatment reduced BPD morbidity and mortality in low-risk infants more than those in high-risk infants (175). Therefore, vitamin A treatment should not be restricted to more than those in high-risk neonates. Craig et al. discovered that nebulized vitamin A is more effective than injectable vitamin A in reducing hyperoxia-induced lung injury and improving BPD treatment (176). Human milk is also rich in vitamin A; therefore, it can exert a therapeutic effect through intestinal pathways.

Vitamin E

Vitamin E is a powerful antioxidant that eliminates free radicals from the human body. Human milk contains alpha- and gamma-tocopherol vitamin E. While infant vitamin E oral preparations are well tolerated throughout pregnancy, several investigations have found that vitamin E levels at birth are much lower in infants with BPD than in normal infants. Vitamin E insufficiency is inversely related to the period in which premature newborns require supplemental breathing, demonstrating an

association between vitamin E deficiency and BPD severity (177). Most vitamin E clinical trials were halted in the 1990s, with several trials concluding that vitamin E had no evident clinical therapeutic effect on BPD. Due to the limitations of the research circumstances at the time and other considerations, such as other early diseases and understanding of BPD, the clinical application of vitamin E has not been extensively recognized (178–181).

However, compared to 30 years ago, qualitative changes in the understanding of BPD have occurred, namely, the revision from old BPD definition to the new BPD definition. With the improvement of premature infant nursing technology, birth gestational age has been raised from 30 weeks to around 26 weeks for children with BPD. At this time, it is in the tubular lung development or early capsule stage. The clinical characteristics of the new BPD were also altered from those of old BPD—"significant lung injury" was revised to "lung developmental arrest." In the contemporary BPD context, it may be argued that research on vitamin E treatment for BPD has lost clinical importance. However, vitamin E supplementation has recently been shown to have a preventive effect on new BPD. BPD can be reduced by increasing the amount of vitamin E in the diet (182).

Phytochemicals

Phytochemicals are a broad range of biochemical molecules produced by plants, but not by the human body. Lactating women can obtain phytochemicals through their diet, resulting in the production of breast milk phytochemicals. Human milk phytochemicals contain polyphenols and carotenoids, which have been shown to have strong antioxidant properties (183).

Polyphenols

Polyphenols are secondary metabolites with a polyphenolic structure found in plants. Flavonoids are an important subgroup of polyphenols, but there are no sources of polyphenols in the body. The amount of polyphenols in breast milk depends on the mother's food intake and absorption (184). Song et al. found epicatechin, epicatechin gallate, epicatechin gallate, naringin, kaempferol, hesperidin, and quercetin in the breast milk of full-term mothers (114).

Polyphenols, which are powerful antioxidants, have been shown to impact signal transmission and modulate intestinal enzyme activity through redox reactions. Polyphenol levels *in vivo* are inversely proportional to MDA, a lipid peroxidation product, implying that polyphenols can mitigate the effects of neonatal lipid peroxidation. Poniedziaek et al. discovered that nursing mothers who eat more vegetables had more polyphenols in their milk and higher antioxidant capacity (185). Currently, researchers are paying increasing attention to the antioxidant ability of polyphenols and flavonoids to avoid OS induced by ROS accumulation and diseases associated with OS. Polyphenol supplementation reduces chronic oxidative cell damage, DNA damage, inflammation, infection, and neurodegenerative disorders (186). Flavonoids have three main antioxidant effects— (1) metal chelation through different flavonoid structures under specific PH values, (2) reducing free radicals (superoxide, hydrogen peroxide, alkoxy) generated through hydrogen donation, where the newly generated free radicals react with other free radicals to form stable quinone structures, and (3) inhibiting oxidases (such as xanthine oxidase, microsomal oxygenase, and lipoxygenase) (187). Supplementing polyphenols in breast milk is a convenient and safe way to absorb them, but there is currently limited research on neonatal plasma polyphenol levels. And there is no evidence that polyphenols have positive effects for development of neonatal lungs, long-term clinical trials are indispensable to determine the benefits of polyphenols for newborns.

Carotenoids

Carotenoids are phytochemicals with antioxidant properties that can reduce the risk of cancer and cardiovascular disease. They are essential for immunological modulation and anti-aging. Beta-carotene can also be converted to the most common form of vitamin A in the body. Carotenoids and vitamin A levels in children with BPD are lower than expected (188). Carotenoid concentrations in breast milk change significantly over time, with α-carotene, β-carotene, alpha-cryptoxanthin, β-cryptoxanthin, zeaxanthin, lutein, and lycopene decreasing during early lactation (1-4 weeks) and then during the following nine weeks. As carotenoids are produced in breast milk during lactation and are consumed continuously after lactation, they do not accumulate in the mother and require exogenous supplementation (114). Given the high antioxidant capacity and ability of carotenoids to increase vitamin A levels in the body, it is reasonable to conclude that carotenoid supplementation is beneficial in treating BPD. Premature newborns can be administered carotenoids in human milk to boost their antioxidant capacity. However, clinical evidence of the advantages of carotenoids in newborn development and their impact on BPD is currently lacking.

Melatonin

Melatonin is a hormone secreted by and retained in the pineal gland and subsequently processed in the liver. Melatonin secretion exhibits a distinct nocturnal circadian pattern. Melatonin is present in breast milk at higher concentrations at night and at lower concentrations during the day. Melatonin is a powerful endogenous antioxidant that can directly remove excess free radicals and promote the expression of other antioxidants such as SOD, CAT, and GPX (189). According to Li et al., melatonin can enhance the oxidative balance in mice with hyperoxia-induced lung injury by lowering MPO, nitrite/nitrate, and MDA levels; increasing GPX, CAT, and SOD activities; and reducing alveolar simplification and interstitial fibrosis. Given the similar lung development in mice and humans, it is plausible to assume that melatonin may protect against newborn hyperoxic lung injury (190). Selami et al. confirmed the therapeutic benefits of melatonin in rats with hyperoxia-induced lung damage. Melatonin treatment was found to increase the expression of lamellar protein and radiate alveolar count produced by AEC II, increase the levels of GPX and SOD, and decrease the levels of MDA, implying that melatonin can regulate the oxidative balance, protect pulmonary vascular endothelial cells, and promote alveolarization in hyperoxia-induced lung injury (191). For premature infants, melatonin treatment could

reduce the mortality and hospital stay of BPD, which also need further studies to identify direct benefits of melatonin (125).

Trace Elements

Trace elements are elements in the human body that contains < 0.005–0.01% of body mass. Copper, selenium, iron, zinc, and other trace elements found in human milk form the foundation of the oxidation reaction of the body. Trace element supplementation can boost antioxidant function by catalyzing multiple redox processes by varying valence and activating endogenous antioxidants to eliminate ROS. Premature newborns acquire vital vitamins from human milk to fight excess reactive radicals. Trace elements are required by the body's antioxidant system; for example, selenium is necessary to maintain the activity of glutathione peroxidase, which is a powerful antioxidant that can remove lipid peroxidation. Selenium deficiency impairs the antioxidant function in the body. Supplementing premature newborns with selenium may improve their BPD prognosis, but it does not prevent BPD (192).

Free Amino Acids

Free amino acids are amino acids that do not form peptides. Human milk contains a range of free amino acids, including arginine, a critical amino acid for premature newborns. Arginine is involved in the circulation of ornithine, which is crucial for immunological function and acid–base balance. The antioxidant properties of arginine are of interest. L-arginine has been shown to lower OS in the exercise state through NO production (193). The combined use of inhaled NO and vitamin A supplementation can minimize morbidity and mortality in premature infants who require early mechanical ventilation (194). *In vivo*, arginine is the raw material for GSH synthesis, and L-arginine supplementation can enhance GSH production and activate the Nrf2–ARE pathway, resulting in endogenous antioxidant responses (195).

Glutamine is a conditionally essential amino acid that can help build muscles, boost the immune system, and expand antioxidant capacity. Exogenous glutamine supplementation for a short period can increase serum CAT and SOD activities and has an antioxidant effect. Glutamine has been shown in some studies to protect mice from hyperoxia-induced lung injury. Arginine–glutamine dipeptide is a stable source of water-soluble glutamine and protects newborn mice from hyperoxic lung injury (196). But there are not direct researches to study the benefits of free amino acids in BPD.

Antioxidant Enzymes and GSH

Antioxidant enzymes are important substances for human body to against OS. Human milk contains antioxidant enzymes such as SOD, CAT, and GPX, which are crucial for preventing lung damage in neonates. SOD catalyzes the conversion of superoxide anion free radicals to hydrogen peroxide. In contrast, CAT and GPX are responsible for hydrogen peroxide breakdown and prevention of oxidative free radical accumulation in the body. Excess ROS cannot be removed in premature newborns due to abnormalities in the endogenous antioxidant enzyme system, leading to ROS accumulation and, ultimately, OS. Breastfeeding can improve the antioxidant capacity of premature infants. Colostrum has a higher antioxidant capacity than transitional milk or mature milk, and it can aid in the early transition from intrauterine hypoxia to extrauterine hyperoxia and improve the antioxidant system (189).

Human milk also contains GSH, a crucial non-enzymatic antioxidant. GSH participates in the circulation of vitamins C and E, boosting antioxidant capacity and engaging in several redox processes as a reducing agent. GSH levels in the alveolar lavage fluid of children with BPD have been shown to be considerably lower than those in children without BPD. A survey of GPX-deficient neonatal mice revealed that increased GSH-dependent redox reactions could lower oxidative lung damage and the prevalence of BPD (71).

DISCUSSION

BPD is a chronic respiratory condition with long-term detrimental implications for newborn health, such as reduced quality of life and poor clinical outcomes. Awareness of BPD has grown in recent years due to ongoing research on its etiology and pathogenesis. OS due to hyperoxia exposure is a significant risk factor for BPD. There are additional preventative and therapeutic targets for BPD. Antioxidant treatment has become an essential strategy for the adjuvant treatment of preterm newborns with BPD despite the lack of standardized clinical signs. OS has a range of effects on normal lung development. When premature infants are born, their lungs are still in the growth stage, and hyperoxia causes poor lung development, increases death of alveolar epithelial cells, and triggers pulmonary vascular remodeling. Although OS plays a role in the development of BPD, there are several limitations to clinical oxidation treatment—treatment is limited to a single type of antioxidant in clinical practice, antioxidant treatment is used only as a supplement to conventional treatment of BPD, and a poor understanding of antioxidant treatment level. However, studies on the degree of antioxidant therapy in preterm newborns using exogenous antioxidants are scarce. Determining how to target the OS site to administer exogenous antioxidant molecules is challenging, which directly impacts the stability and efficacy of antioxidant therapies. Therefore, clinical antioxidant therapy often does not have the desired effect. Antioxidant treatment is undeniably effective in combating oxidative imbalances. Inhibition of OS *via* the Nrf2, SIRT1, and TXN signaling pathways has shown promising results in animal models, demonstrating the potential of antioxidant therapy in preventing and controlling BPD. More clinical trials are needed to confirm the association between OS and BPD and further investigate the benefits of antioxidant therapy for BPD.

Human milk is the most natural and safe food for infants. The current review reveals that breastfeeding may help minimize the risk of developing BPD, although the role of human milk in preventing and treating BPD is unknown. Breastfeeding has many beneficial effects on BPD. Human milk delivers a high level of nutrients that aid in the normal development of premature newborns after birth, improve their nutritional status, and encourage the continuous development of their lungs.

Furthermore, breastfeeding reduces postpartum inflammation, improves immunity, reduces newborn infections, and lowers the incidence of BPD. Human milk also contains several antioxidants that are thought to minimize OS in the BPD process. In this review, we examined several active compounds in human milk, considering them from different perspectives to collate how they may ameliorate BPD OS in lung development as follows: 1. direct secretion of antioxidant substances clears free radicals and prevents their accumulation, 2. metal chelation, 3. enhancement of the antioxidant signaling pathway, 4. interaction with the intestinal flora, 5. inhibition of lipid peroxidation, and 6. anti-inflammatory and anti-infection properties.

Human milk has a high antioxidant capacity, which supports the benefits of breastfeeding for preterm neonates. However, there are several limitations to this view. Firstly, we identify only the antioxidants that play an antioxidant role in BPD. It is unclear whether they act as effective antioxidants in breast milk and whether there is a synergistic effect between these substances. Secondly, most studies cited in this view associated with the antioxidant effect of BPD do not come from breast milk OS. The research about breast milk antioxidants and their effects on BPD are still limited based on ethical factors. thirdly, there are limited data on human milk from mothers of premature infants; therefore, differences in antioxidant capacity compared to that of human milk from mothers of full-term infants has not been clearly shown. Owing to ethical considerations, few randomized controlled trials to prevent premature BPD have been conducted. Hence, observational studies have been given precedence.

While the effects of breast milk on BPD have been addressed, there are much research in this territory that require future studies. In this view, we suggest researchers take these issues into account: 1. Should the combination of probiotics be added to the formula? 2. Does the antioxidants in breast milk have synergistic effects when breastfeeding? 3. What are the positive effects of MFGM on antioxidation? 4. What roles does the gut-lung axis play when SCFAs affect as antioxidative substances? 5. Do the Vitamin E treatments have effects accompanied by the evolution of BPD? 6. How does the addition of polyphenols and carotenoids to the formula impact the development of neonatal lungs? 7. Does melatonin have effects on BPD in preterm infants? These controversies require further clinical studies although the limitations are still hard. And we also noticed that stem cells in breast milk present a prominent potential for BPD treatment, it is worth studying their antioxidative effects on BPD.

Individual variables related to human milk, such as neonatal birth gestational age, whether the mother is breastfeeding, and whether exclusive breastfeeding is practiced, can have a significant impact on the composition of human milk. Human milk from mothers of premature newborns differs substantially from human milk from mothers of full-term newborns. Chrustek et al. found that compared to human milk from mothers of full-term newborns, that from mothers of premature infants may contain more antioxidants. Premature newborns with a higher total antioxidant status are protected from free radicals, preventing ROS accumulation and OS (197). Antioxidants in human milk have been modified to the PMA to provide improved protection for premature newborns. Some active compounds in breast milk can increase the antioxidant capacity of premature infants, which is conducive to the rapid establishment of oxidative balance in premature infants with insufficient antioxidant capacity.

CONCLUSION

Human milk contains many bioactive compounds that supply essential nutrients to newborns and act as antioxidants to protect premature babies from OS. Existing research suggests that breastfeeding can ameliorate the effects of BPD. However, more high-quality tests and studies are needed to provide evidence for the role of breastfeeding in the prevention and control of BPD. Breastfeeding is recommended for full-term and preterm infants, although their antioxidant capacities differ. High-dose breastfeeding has the potential to be an effective and inexpensive treatment option for BPD.

AUTHOR CONTRIBUTIONS

XY is responsible for the collection of data and writing of the original manuscript. SJ is responsible for the organization of the original manuscript. XD and ZL are responsible for editing. AC and RY are responsible for the concept development, revision, review of the manuscript, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Lifestyle intervention during pregnancy in patients with gestational diabetes mellitus and the risk of neonatal hypoglycemia: A systematic review and meta-analysis

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Objective: Neonatal hypoglycemia is a severe adverse consequence of infants born to mothers with gestational diabetes mellitus (GDM), which can lead to neonatal mortality, permanent neurological consequences, and epilepsy. This systematic review and meta-analysis of randomized controlled trials (RCTs) was conducted to explore the effect of lifestyle intervention during pregnancy in women with GDM on the risk of neonatal hypoglycemia.

Methods: PubMed, Web of Science, Cochrane Library, CINAHL, and SPORTDiscus databases were searched by 1st April 2022. Data were pooled as the risk ratio (RR) with 95% CIs of neonatal hypoglycemia. Random-effects, subgroup analyses, meta-regression analysis, and leave-one-out analysis were conducted, involving 18 RCTs.

Results: Prenatal lifestyle intervention could significantly reduce the risk of neonatal hypoglycemia (RR: 0.73, 95% CI: 0.54–0.98, $P = 0.037$). Subgroup analysis further demonstrated that the reduced risk of neonatal hypoglycemia was observed only when subjects were younger than 30 years, initiated before the third trimester, and with dietary intervention. Meta-regression analysis revealed that the risk of neonatal hypoglycemia post lifestyle intervention was lower in mothers with lower fasting glucose levels at trial entry.

Conclusion: We found that prenatal lifestyle intervention in women with GDM significantly reduced the risk of neonatal hypoglycemia. Only lifestyle intervention before the third trimester of pregnancy, or dietary intervention only could effectively reduce the risk of neonatal hypoglycemia. Future studies are required to explore the best pattern of lifestyle intervention and to

determine the proper diagnostic criteria of GDM in the first/second trimester of pregnancy.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/#myprospero, PROSPERO, identifier: CRD42021272985.

KEYWORDS

lifestyle intervention, gestational diabetes mellitus, neonatal hypoglycemia, systematic review, meta-analysis

Introduction

Gestational diabetes mellitus (GDM), defined as hyperglycemia with onset or first recognized during pregnancy, is associated with an increased risk of a range of adverse outcomes for offspring that can be passed on from generation to generation (1), namely, hypoglycemia (2), obesity, and type 2 diabetes (3–5). The incidence rate of GDM ranged from 3.4% to 37.7%, depending on different diagnostic criteria and population (6). A population-based cohort study showed that infants born to mothers with GDM had a significantly increased risk of hypoglycemia (OR: 11.71, 95% CI: 7.49–18.30) (7). The high concentration of blood glucose in the mother leads to an increase in fetal glucose intake, which stimulates excess fetal insulin secretion, thereby inducing neonatal hypoglycemia (8). Neonatal hypoglycemia is an important factor in neonatal mortality (9) and permanent neurological consequences (9, 10). Even infants who are slightly and transiently exposed to hypoglycemia are at risk of later delayed neurodevelopment (11–15). Severe neonatal hypoglycemia can also lead to epilepsy, personality disorder, impaired heart function, and muscle weakness (9).

Lifestyle interventions, mainly dietary interventions, also including physical exercise interventions and other interventions, are usually the first-line strategy for managing GDM (16). A systematic review and meta-analysis of 19 controlled trials showed that dietary intervention during pregnancy effectively reduced the incidence rate of GDM (17). After being diagnosed with GDM, 70%−85% of patients were efficient to control blood glucose by lifestyle intervention *per se* according to American Diabetes Association (18). Studies have shown that the adverse perinatal outcomes caused by GDM might be improved with the treatment of GDM (19). Despite that, the effect of prenatal lifestyle intervention on the incidence of neonatal hypoglycemia remains inconclusive, although mounting studies have sprung up. Some results from human studies of randomized controlled trials (RCTs) suggested that lifestyle intervention could effectively reduce the risk of neonatal hypoglycemia (20–22). Recently, a large well-conducted RCTs revealed that the overall neonatal complications were significantly reduced by 47% post-smartphone-based lifestyle intervention (23). While some other evidences from human RCTs reported null effects on the risk of neonatal hypoglycemia (24–26).

Thus, the purpose of this review was to evaluate the effect of lifestyle intervention during pregnancy in women with GDM on the hypoglycemia risk of their neonate and to examine related influencing factors.

Methods

Literature search

This meta-analysis was carried out in compliance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA 2020) guidelines (27). The protocol number of this study is PROSPERO CRD 42021272985. Relevant articles published through 1st April 2022 were searched from PubMed, Web of Science, Cochrane Library, Cumulated Index to Nursing and Allied Health Literature (CINAHL), and SPORTDiscus (a full-text database of sports and sports medicine journals in the EBSCOhost), based on the Population, Intervention, Comparator, Outcome and Study design (PICOS) framework. The following search strategy was used: (lifestyle OR diet OR "physical exercise") AND (pregnancy OR "diabetes mellitus") AND "neonatal hypoglycemia" AND "randomized controlled trial." Detailed search terms are given in Supplementary Table 1.

Study selection

Two authors (YHW and HHZ) independently screened titles and abstracts, then reviewed the full text of all relevant studies for eligibility. The third researcher (YZ OR ZZ) arbitrated any discrepancies to reach a consensus. We included RCTs that evaluated the effect of lifestyle intervention during pregnancy on patients diagnosed with GDM with reported neonatal hypoglycemia. Inclusion criteria for considering studies for this review were: (1) types of studies: published randomized controlled trials; (2) types of participants: pregnant women diagnosed with GDM (defined by trialists); (3) types of interventions: lifestyle interventions (dietary intervention with

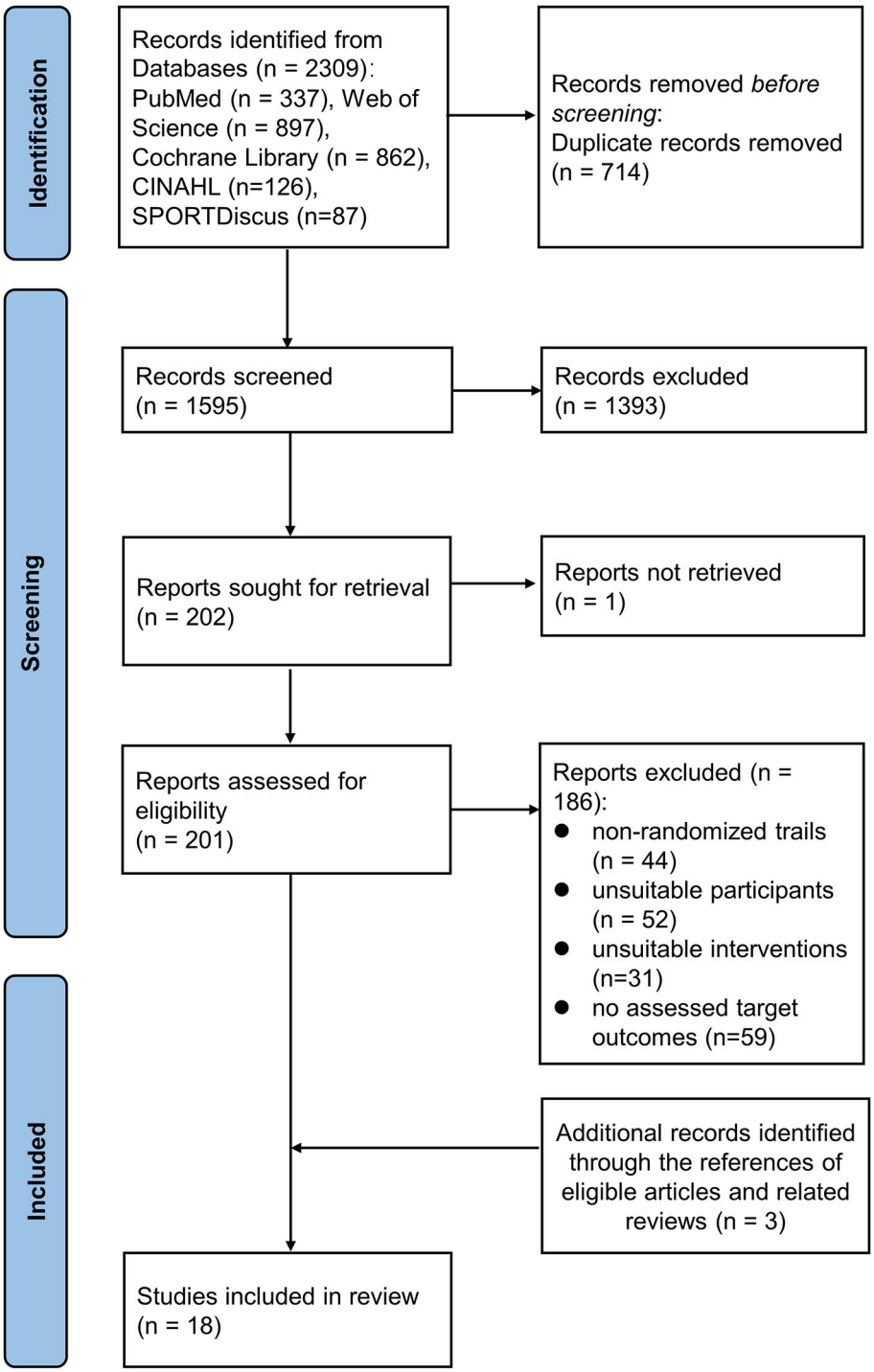

FIGURE 1
Flowchart of study selection process through the review.

or without those following interventions: exercise intervention, health education, self-monitoring of blood glucose, etc.) vs. placebo or usual care; and (4) types of outcome measures: neonatal hypoglycemia. The exclusion criteria included: (1) types of studies: quasirandomized trials or animal studies or reviews; (2) types of participants: patients with type 1 or type 2 diabetes before pregnancy, healthy subjects; (3) types of intervention: comparing different lifestyle interventions; and (4) types of outcome measures: lacking the results of neonatal hypoglycemia or sufficient data to calculate the results of

neonatal hypoglycemia. We also conducted a manual search for reference lists of the included articles.

Data extraction and quality assessment

Data extraction and quality assessment were conducted by two trained people (YHW and HHZ) independently. The data were extracted to a form we designed, including: the first author's surname, publication year, study design, study location, sample size (intervention/comparators), age at pregnancy, gestational age at baseline, mother's fasting glucose level at baseline, maternal body mass index (BMI) (kg/m^2) at baseline or prepregnancy, intervention information, comparator information, and the outcome of neonatal hypoglycemia. Studies containing two or more intervention strata were analyzed as separate trials. The risk of bias (RoB) of each included study was assessed according to the criteria of the Cochrane Handbook for Systematic Reviews of Interventions (28). The Grading of Recommendation, Assessment, Development, and Evaluation (GRADE) approach was used to assess the quality of evidence (29). According to the GRADE handbook, study design dictates the baseline quality of the evidence (RCTs are initially assigned a ranking of high), and other factors could downgrade (risk of bias, inconsistency, indirectness of evidence, imprecision, and publication bias) or upgrade (large effect size, plausible residual confounding, and dose-response relationship) the quality of evidence. Discrepancies were resolved through discussion or by involving the third reviewer (YZ, OR, and ZZ).

Statistical analysis

A fixed-effect meta-analysis was used to pool estimates of summary risk ratio (RR) with 95% CIs of neonatal hypoglycemia. If substantial statistical heterogeneity was detected, the random-effects model was used to summarize the overall effect. Advanced data extraction was performed for studies that did not directly provide the mean and SD of continuous variables (30). The heterogeneity among studies was tested using the chi^2 test and quantified by I^2-statistic (31). The presence of heterogeneity was indicated by P-value < 0.10 in the chi^2 test or $I^2 > 30\%$.

Sources of potential heterogeneity were investigated by subgroup analyses and meta-regression based on age at pregnancy, gestational age at baseline, maternal fasting glucose level at baseline, and intervention types. The P-value < 0.1 was considered statistically significant in meta-regression analysis. Sensitivity analyses based on leave-one-out cross-validation were conducted to assess the robustness of the results in primary meta-analyses and to evaluate the impact of each trial on the heterogeneity, using a p-value < 0.05 as the criterion (32). Begg's and Egger's regression tests and funnel plots were used to assess possible publication bias. The P-value < 0.10 suggested the presence of publication bias (33). If publication bias was found, the trim and fill method was utilized (34).

Data analyses were performed using STATA version 11.0 (Stata Corp, College Station, TX, USA), with double data input to avoid input errors. The risk of bias in included studies was assessed using RevMan version 5.3 (Cochrane Collaboration, Oxford, UK). $P < 0.05$ was deemed as statistically significant unless specified elsewhere.

Results

Literature search and study characteristics

As shown in Figure 1, the detailed process of literature search and study selection is presented in the flowchart. A total of 2,309 articles (337 from PubMed, 897 from Web of Science, 862 from Cochrane Library, 126 from CINAHL, and 87 from SPORTDiscus) were identified through initial searching, out of which 714 studies were removed because of duplication. Then 1,393 records were excluded after screening the title/abstract. The remaining 186 articles were eliminated for the following reasons: 44 studies were excluded due to inappropriate article design, 52 studies did not meet the inclusion criteria due to unsuitable participants, 31 studies lacked proper treatment, and 59 studies lacked sufficient data. Additional 3 studies were included through evaluating the reference lists of the included articles. Eventually, 18 eligible studies were included in the final quantitative synthesis.

Characteristics of included studies

Characteristics of 18 studies (19–26, 35–44) included in this meta-analysis are shown in Table 1, involving a total of 5,182 women and 4,945 newborns. Sample sizes ranged from 45 (35) to 1,030 (19) newborns. 13 studies (20, 21, 24, 25, 35–42, 44) had a sample size of fewer than 300 newborns. Five studies were conducted in China (21, 22, 24, 36, 43), four in Iran (25, 35, 38, 44), two each in the United States (26, 42) and Australia (19, 41), and one each in the United Arab Emirates (20), Canada (37), Egypt (39), the United Kingdom (40), and Singapore (23). All studies reported data for maternal age. The mean maternal age of the intervention group ranged from 26.88 ± 3.15 (21) to 31.70 ± 4.00 (23) years. In the control group, the mean maternal age ranged from 26.20 ± 3.10 (44) to 32.20 ± 4.40 (23) years. Studies were initiated in the first ($n = 3$) (36, 39, 43), second ($n = 6$) (21, 23, 25, 35, 38, 40), and third ($n = 6$) (19, 22, 24, 26, 41, 42) trimesters, while three studies (20,

TABLE 1 Characteristics of included studies in this meta-analysis (18 studies).

Study	Design	Country	Sample (intervention/ comparators)	Age at pregnancy	Gestational age at baseline (weeks)	Fasting glucose level at baseline (mmol/L)	Interventions	Comparators
Asemi et al. (35)	RP, Db	Iran	Mothers: 22/23 Newborns: 22/23	30.95	25.56	>5.23	VD supplements (50,000 IU VD3 pearl 2 times: at study baseline and day 21 of intervention)	Placebo (2 placebos at the mentioned times)
Cao et al. (24)	RP	China	Mothers: 127/148 Newborns: 127/148	30.39	30.46	4.79	Comprehensive intensive therapy (individualized diabetes education, lifestyle intervention, scheduled clinic visits, strict glucose control, and frequent glucose self-monitoring)	Standard therapeutic regimen (group education and instruction the importance of proper diet, exercise, and self-monitoring of glucose level)
Crowther et al. (19)	RP, Db	Australia	Mothers: 490/510 Newborns: 506/524	30.49	29.12	4.80	Individualized dietary advice, blood glucose monitoring +/- insulin therapy	Usual care
Elnour et al. (20)	RP	United Arab Emirates	Mothers: 99/66 Newborns: 99/66	30.94	8–19	/	Structured pharmaceutical care, structured education on diet, exercise and diabetes treatment, self-monitoring of blood glucose	Traditional services (monthly clinic visits and self-monitoring of plasma glucose)
Gao et al. (36)	RP, Db	China	Mothers: 123/121 Newborns: 123/121	30.64	10.00	5.71	Phytosterol-enriched spreads, 20g/day, contains 4 g of phytosterols/day	Regular margarine spread, 20g/day
Garner et al. (37)	DP, Sb	Canada	Mothers: 149/150 Newborns: 149/150	30.70	24–32	10.05	Calorie–restricted diet of 35 kcal/kg ideal body weight per day	Unrestricted healthy diet
Jamilian et al. (25)	RP, Db	Iran	Mothers: 30/28 Newborns: 30/28	29.38	24–28	5.27	VD (50,000 IU/every 2weeks) + probiotic (8 × 109 CFU/day)	Placebo
Jamilian et al. (25)	RP, Db	Iran	Mothers: 29/28 Newborns: 29/28	30.56	24–28	5.30	probiotic (8 × 109 CFU/day)	Placebo
Jamilian et al. (38)	RP, Db	Iran	Mothers: 30/30 Newborns: 29/30	30.05	26.10	>5.11	1,000 mg omega-3 fatty acids from flaxseed oil + 400 IU VE supplements	Placebo
Karamali et al. (38)	RP, Db	Iran	Mothers: 30/30 Newborns: 30/30	26.70	/	/	Synbiotic capsule containing Lactobacillus acidophilus strain T16 (IBRC-M10785), L. casei strain T2 (IBRC-M10783), and Bifidobacterium bifidum strain T1 (IBRC-M10771) (2 × 109CFU/g each) + 800 mg inulin (HPX)	Placebo

(Continued)

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TABLE 1 Continued

Study	Design	Country	Sample (intervention/ comparators)	Age at pregnancy	Gestational age at baseline (weeks)	Fasting glucose level at baseline (mmol/L)	Interventions	Comparators
Landon et al. (26)	RP	USA	Mothers: 485/473 Newborns: 381/357	29.05	28.85	4.80	Nutritional counseling and diet therapy +/- insulin plus self-monitoring of blood glucose	Usual care +/- insulin plus self-monitoring of blood glucose
Maged et al. (39)	RP, Sb	Egypt	Mothers: 100/100 Newborns: 100/100	27.40	10–12	5.06	1 g L-ascorbic acid/day	Placebo
Meng et al. (21)	RP	China	Mothers: 45/48 Newborns: 45/48	26.88	24.12	5.04	Comprehensive nursing intervention (psychological intervention, health education, diet control, exercise intervention, pregnancy monitoring, and prevention of postpartum complications)	Routine nursing (one-off health education and nutrition and exercise guidance, regular pregnancy monitoring, regular postpartum care)
Min et al. (40)	RP, Db	UK	Mothers: 67/71 Newborns: 58/56	32.25	26.85	5.55	2 capsules of DHA-enriched formula/day	2 capsules of high oleic acid sunflower seed oil/day
Rae et al. (41)	RP, Db	Australia	Mothers: 66/58 Newborns: 59/50	30.39	28.19	4.85	Moderately energy restricted diabetic diet providing between 6,800 and 7,600 kJ/day	Diabetic diet which was not energy restricted, providing approximately 8,600–9,500 kJ/day
Trout et al. (42)	RP	USA	Mothers: 37/31 Newborns: 37/31	28.88	29.78	5.07	Lower-carbohydrate diet (35–40% of total calories)	Usual pregnancy diet (50–55% carbohydrate)
Yang et al. (43)	RP, Db	China	Mothers: 339/361 Newborns: 339/361	29.80	10.80	5.05	Shared care (Individualized dietary and physical activity counseling, energy intakes recommendation, moderate physical activity daily, self-monitoring blood glucose +/-insulin)	Usual care (hospital-based education session +/- insulin)
Yew et al. (23)	RP, Sb	Singapore	Mothers: 170/170 Newborns: 168/165	31.95	26.85	4.65	Usual care + Habits-GDM app (integrated dietary, physical activity, weight, and glucose monitoring)	Usual care (hospital-based education session, self-monitoring of blood glucose +/- insulin)
Zhang et al. (22)	RP, Db	China	Mothers: 176/150 Newborns: 176/175	29.19	29.00	5.81	500 mg of EGCG/day	Placebo

Db, double blind; DHA, docosahexaenoic acid; EGCG, epigallocatechin 3-gallate; RC, randomized crossover; RP, randomized-parallel; Sb, single blind; UK, United Kingdom; USA, United States of America, VC, vitamin C; VD, vitamin D; VE, vitamin E.

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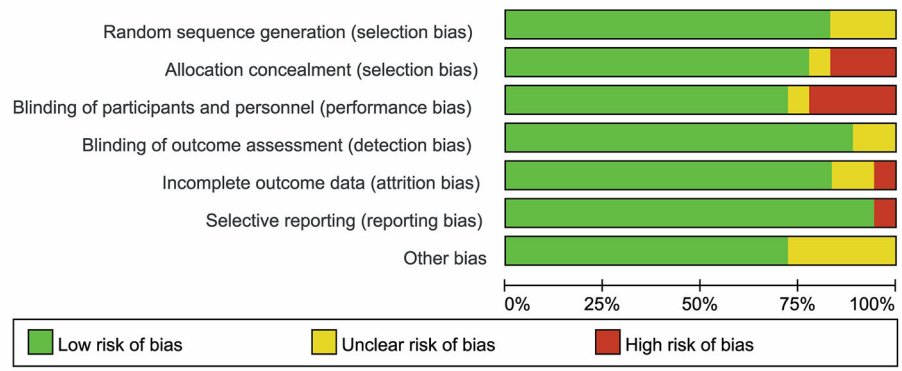

FIGURE 2
Risk of bias of summary was assessed using the risk of bias (RoB) tool of the Cochrane Handbook for Systematic Reviews of Interventions.

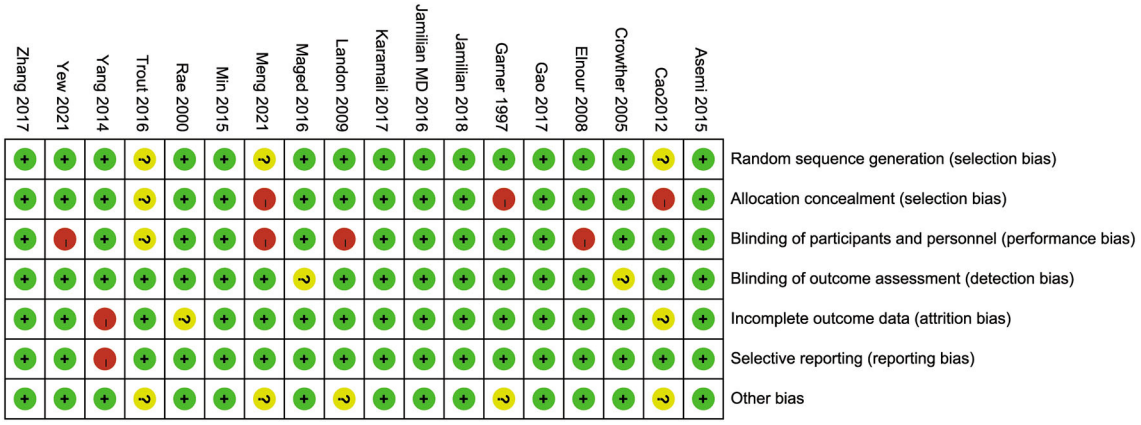

FIGURE 3
Risk of bias of each included study was assessed using the risk of bias (RoB) tool of the Cochrane Handbook for Systematic Reviews of Interventions.

37, 44) were unspecified. Specific gestational age at trial entry was reported in 13 studies (19, 21–24, 26, 35, 36, 38, 40–43), with a mean age ranging from 10.00 ± 0.32 (36) to 30.46 ± 4.71 (24) weeks. The specific fasting glucose level at trial entry was reported in 14 studies (19, 21–26, 36, 37, 39–43), with a mean level ranging from 4.65 ± 0.50 (23) to 10.05 ± 1.61 (37) mmol/L. Maternal BMI at trial entry and before pregnancy was reported in eight studies (19, 24, 25, 38, 39, 41, 42, 44) and seven studies (22, 23, 26, 35, 36, 40, 43), respectively. As for treatments of the intervention groups, the comprehensive intervention was utilized in seven studies (19–21, 23, 24, 26, 43), a calorie/carbohydrate-restricted diet was utilized in three studies (37, 41, 42), vitamin D supplements were utilized in two studies (25, 35), probiotics were utilized in two studies (25, 44), and phytosterol-enriched food (36), omega-3 fatty acids plus vitamin E supplements (38), vitamin C supplements (39), docosahexaenoic acid (DHA) supplements (40), and epigallocatechin 3-gallate (EGCG) supplements (22) were used in every single study.

Excluded studies

In total, six studies (45–50) included subjects without GDM, additional two studies (51, 52) included women with impaired glucose tolerance but did not meet the diagnosis of GDM as defined by trialists. Two studies (53, 54) did not use an intervention/comparison included in this review.

Risk of bias in included studies

As shown in Figures 2, 3, three studies (21, 24, 42) were considered to be of unclear risk of bias for randomization, the

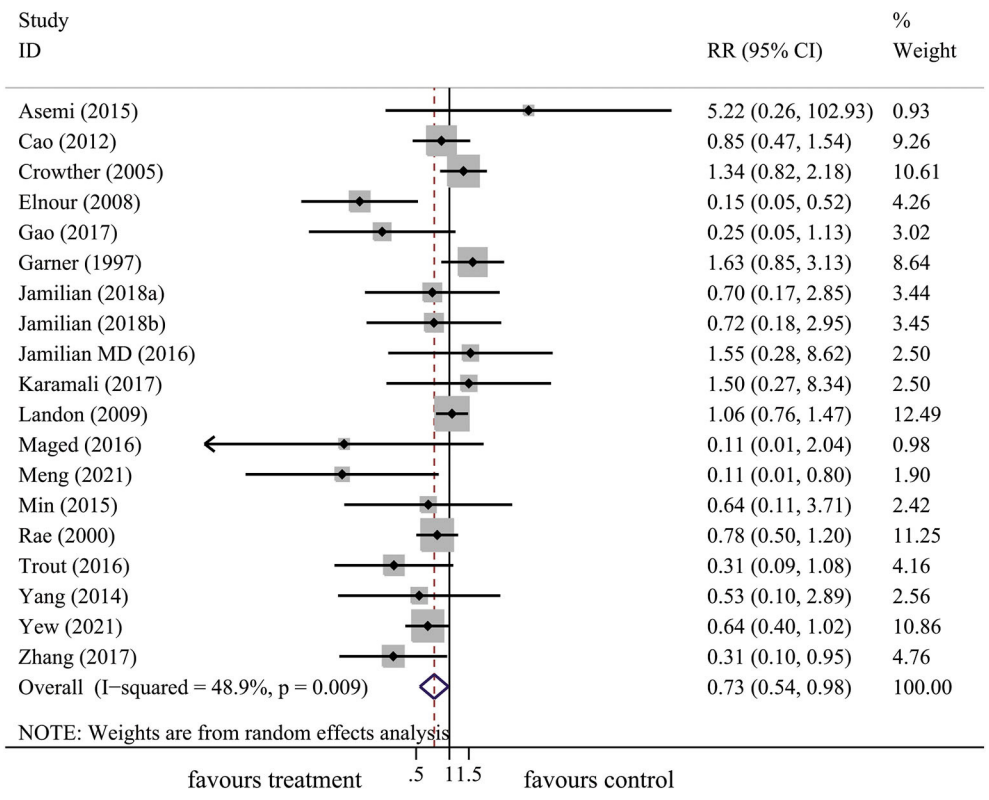

FIGURE 4
The forest plot demonstrated the effect of prenatal lifestyle intervention in women with GDM on the risk of neonatal hypoglycemia by pooling data from 18 studies.

TABLE 2 Results of subgroup analysis and publication bias stratified by study characteristics.

Variables	Trials (*n*)	RR (95% CI)	P^1	Heterogeneity		P^3	
				I^2 (%)	P^2	Begg's value	Egger's value
Overall	19	0.73 (0.54 to 0.98)	**0.037**	48.9	**0.009**	0.529	0.713
Age at pregnancy							
≤ 30 years	8	0.52 (0.27 to 0.99)	**0.046**	51.4	**0.044**	1.000	0.159
> 30 years	11	0.81 (0.57 to 1.17)	0.272	52.3	**0.021**	0.161	0.537
Gestational age at baseline							
< 14 weeks	3	0.30 (0.10 to 0.86)	**0.025**	0.00	0.614	1.000	0.730
14∼28 weeks	7	0.66 (0.44 to 0.98)	**0.039**	0.7	0.419	0.548	0.242
≥ 28 weeks	6	0.85 (0.61 to 1.19)	0.337	50.6	**0.072**	0.260	0.178
Fasting glucose level at baseline							
< 5.1 mmol/L	9	0.79 (0.57 to 1.07)	0.131	48.2	**0.051**	0.602	0.144
≥ 5.1 mmol/L	8	0.78 (0.42 to 1.46)	0.439	39.1	0.118	0.386	0.220
Intervention type							
Dietary intervention only	12	0.69 (0.48 to 0.98)	**0.041**	54.8	**0.011**	0.273	0.915
Dietary + other interventions	2	0.55 (0.04 to 6.83)	0.642	61.1	0.109	0.317	-
Dietary + exercise + other interventions	5	0.80 (0.41 to 1.55)	0.504	50.7	0.087	0.624	0.317

P^1 value for net change; P^2 value for heterogeneity in the subgroup; P^3 value for publication bias; significant p-values are highlighted in bold prints.

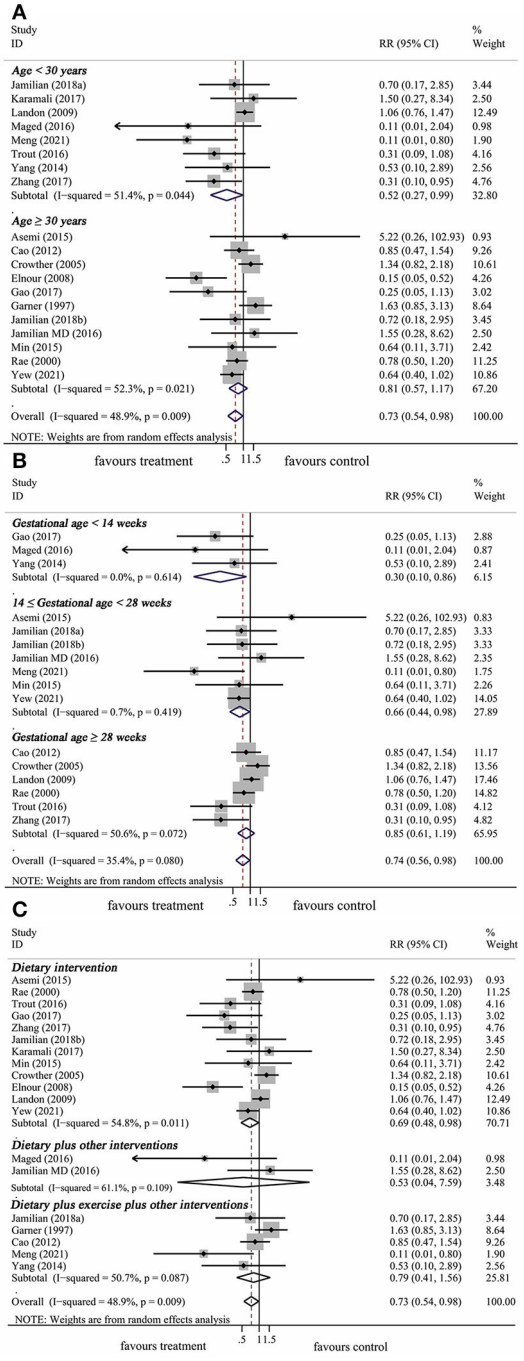

FIGURE 5
Subgroup analysis of prenatal lifestyle intervention on the risk of neonatal hypoglycemia stratified by maternal age at pregnancy **(A)**, gestational age at baseline **(B)**, and intervention types **(C)**.

other 15 (19, 20, 22, 23, 25, 26, 35–41, 43, 44) studies were at low risk of bias. Three studies (21, 24, 37) were considered to be of high risk of bias for allocation concealment, one study (42) was judged to be of unclear risk of bias. Four studies (20, 21, 23, 26) had a high risk of performance bias, one study (42) was judged to be of unclear risk of bias. Two studies (19, 39) had an unclear risk of detection bias. One study (43) was judged as high risk for attrition bias and two studies (24, 41) were considered to be of unclear risk for attrition bias. One study (43) was considered to be of high risk for reporting bias. Five studies (21, 24, 26, 37, 42) had unclear risk of other biases (potential biases related to the study design).

Effect of lifestyle intervention on the risk of neonatal hypoglycemia

In total, 18 articles included 19 trials that explored the effect of lifestyle intervention on the risk of neonatal hypoglycemia. With consideration of the heterogeneity, we used the random-effect model to get pooled estimates. Results from our meta-analysis suggested that lifestyle intervention during pregnancy could significantly reduce the risk of neonatal hypoglycemia (RR: 0.73, 95% CI: 0.54 to 0.98, $P = 0.037$) (Figure 4). The results of the I^2 and chi^2 test demonstrated that there was substantial heterogeneity in the primary meta-analysis ($I^2 = 48.9\%$; $P = 0.009$) (Table 2). The Begg's and Egger's tests indicated no significant publication bias in the primary analysis (Table 2).

Subgroup and meta-regression analysis

The results of the subgroup analysis were summarized in Table 2. The results of subgroup analysis revealed that the effect of lifestyle intervention on the risk of neonatal hypoglycemia was influenced by maternal age at pregnancy and gestational age at trial entry. Lifestyle intervention was associated with a decrease in the risk of neonatal hypoglycemia only in studies with mothers younger than 30 years (RR: 0.52, 95% CI: 0.27–0.99, $P = 0.046$), but not in studies with mothers ≥ 30 years (RR: 0.81, 95% CI: 0.57–1.17, $P = 0.272$) (Figure 5A). Furthermore, significant reductions of risk of neonatal hypoglycemia post lifestyle intervention were observed only in studies with gestational age < 14 weeks (first trimester) at trial entry (RR: 0.30, 95% CI: 0.10–0.86, $P = 0.025$) and in studies with gestational age between 14 and 28 weeks (second trimester) at trial entry (RR: 0.66, 95% CI: 0.44–0.98, $P = 0.039$), but not in studies with gestational age ≥ 28 weeks (third trimester) at trial entry (RR: 0.85, 95% CI: 0.61–1.19, $P = 0.337$) (Figure 5B). No significant effect was observed in the subgroup results of baseline maternal fasting glucose level < 5.1 mmol/L or ≥ 5.1 mmol/L. In addition, results from subgrouping analysis by type of lifestyle intervention presented a reduction of neonatal hypoglycemia risk in studies using dietary intervention only (RR: 0.69, 95% CI: 0.48–0.98, $P = 0.041$), while this effect did not exist in dietary plus other interventions or dietary plus exercise plus

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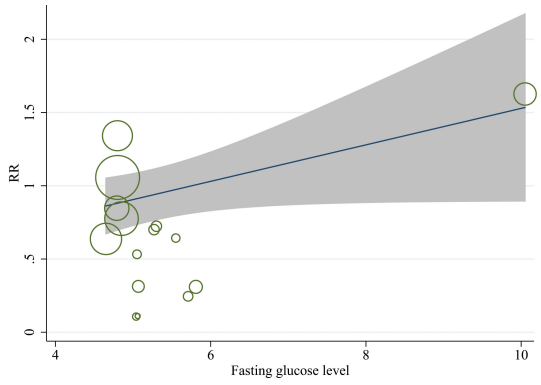

FIGURE 6
Meta-regression model for the effect of prenatal lifestyle intervention on the risk of neonatal hypoglycemia, adjusted for maternal fasting blood glucose levels at baseline.

other interventions (Figure 5C). The Begg's and Egger's tests indicated no significant publication bias in the above subgroup analyses (Table 2).

A meta-regression analysis was conducted to explore the potential sources of heterogeneity. Among selected covariates, including maternal age at pregnancy, gestational age at trial entry, and maternal fasting glucose level at trial entry, the results of meta-regression analysis revealed that maternal fasting glucose level at trial entry was a potential confounder of the effect of lifestyle intervention on the risk of neonatal hypoglycemia, with adjusted R^2 of 45.04% (Table 3 and Figure 6). There was no significant association between the risk ratio and other covariates listed in Table 3.

Sensitivity analysis

Regarding the robustness of overall effect sizes, we performed a leave-one-out cross-validation for sensitivity analysis (Figure 7). The results of the leave-one-out cross-validation suggested that three studies (19, 20, 37) contributed most to the heterogeneity in the primary meta-analysis. After excluding these studies, the pooled results remained significant (RR: 0.71, 95% CI: 0.54–0.93, $P = 0.012$), thus the effect of the intervention on the risk of neonatal hypoglycemia might be underestimated due to heterogeneity between studies.

Evidence level rated by GRADE

According to the GRADE protocol, as shown in Table 4, the evidence level of the overall meta-analysis was at a moderate level because significant heterogeneity existed.

Discussion

All in all, there was "moderate" quality evidence from 18 RCTs indicating that prenatal lifestyle intervention in women with GDM was associated with a 27% decreased risk of having a baby with hypoglycemia. Subgroup analysis further demonstrated that the reduced risk of neonatal hypoglycemia post lifestyle intervention was observed only in studies with subjects younger than 30 years, initiated in the first or second trimester, and with dietary intervention. In addition, meta-regression analysis revealed that maternal fasting glucose levels at trial entry were positively associated with the risk ratio of neonatal hypoglycemia post lifestyle intervention.

To the best of our knowledge, this is the first comprehensive systematic review and meta-analysis that evaluated the effect of prenatal lifestyle intervention in women with GDM on the risk of neonatal hypoglycemia. Unlike our results, relevant systematic reviews to date did not find any significant benefit of lifestyle intervention for neonatal hypoglycemia. The meta-analysis from Cochrane (55) included six studies, that reported no significant association between the lifestyle treatment of GDM and the risk of neonatal hypoglycemia. Five of the six studies were also included in our meta-analysis, but the remaining one study (51) was excluded because the study population was women with impaired glucose tolerance rather than patients with GDM. In addition, a systematic review (56) reported no difference between infants exposed to control or diet and exercise interventions before birth for the risk of hypoglycemia, which was based on two studies with overweight or obese women. Unlike our findings, another meta-analysis (57) based on two studies, which were both included in our review, found that dietary intervention in patients with GDM did not change the neonatal outcome of hypoglycemia. In our present study, we found that only dietary intervention in patients with GDM could reduce the risk of neonatal hypoglycemia, but not dietary intervention plus other interventions. This might be due to dietary advice or counseling (rather than supplements) being the main form of dietary intervention in the subgroups of dietary plus other interventions with or without exercise (one (19) of two (19, 26) studies in the group of dietary plus other interventions and three (20, 24, 43) of five (20, 21, 23, 24, 43) studies in the group of dietary plus exercise plus other interventions applied individualized dietary advice or counseling intervention). Since the small number of studies with other interventions included in our study, future large-scale RCTs are still required to further explore the best pattern of lifestyle intervention during pregnancy to reduce the risk of neonatal hypoglycemia. There is no relevant report describing long-term follow-up of hypoglycemia outcomes after changing lifestyle during pregnancy.

TABLE 3 Meta-regression analysis of potential moderators.

Variables	Trials (*n*)	Coefficient	95% CI	P	Covariate adjusted R^2
Age at pregnancy	19	−0.001	−0.239 to 0.237	0.994	-
Gestational weeks at baseline	16	0.051	−0.032 to 0.135	0.150	-
Fasting glucose level at baseline	15	0.128	−0.027 to 0.283	**0.099**	45.04%
Intervention type	19	0.062	−0.141 to 0.266	0.525	-

Significant p-values are highlighted in bold prints.

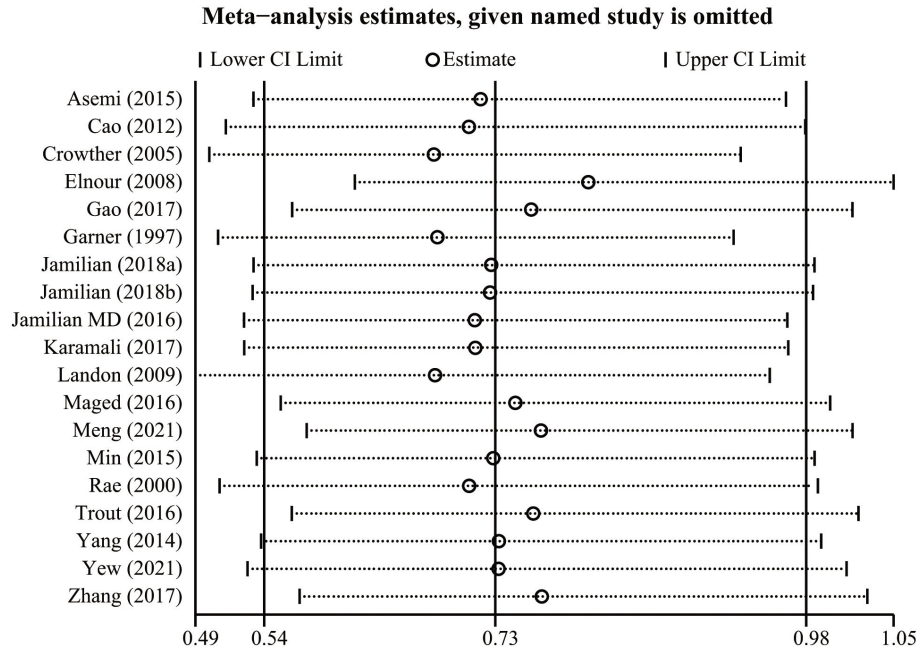

FIGURE 7
The result of leave-one-out cross-validation analysis.

TABLE 4 Grades of Recommendation, Assessment, Development and Evaluation (GRADE) quality of evidence.

Outcome	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication bias	Effect Size	Plausible residual confounding	Dose-response gradient	GRADE rating
	None	serious [a]	None	None	none	none	none	None	Moderate

Significant and unexplained variability exists in the primary meta-analysis.

The role of lifestyle intervention has been greatly appreciated as a clinical treatment in GDM (58). In our present meta-analysis, prenatal lifestyle intervention resulted in a significant reduction in the risk of neonatal hypoglycemia. Achieving glycemic control in women with GDM is critical for reducing the risk of neonatal hypoglycemia (59). Notably, the glucose threshold recommended by recent guidelines (1, 60, 61) is lower than previously recommended (62, 63) for the diagnosis of GDM. Therefore, less severe hyperglycemia has been classified as GDM in recent years, which is also conducive to the effect of lifestyle intervention. Maternal age is known to affect the outcomes of pregnancies (64). Recently, a meta-analysis of 24 studies showed that the risk of GDM increased by 7.90% with each-one year increase in maternal age from 18 (65). The increase in maternal age is also related to the incidence of macrosomia (66), small for gestational age (67), and cesarean section (68), which are all independent risk factors for neonatal hypoglycemia (69). Consequently, we postulated that the adverse

outcomes associated with advanced maternal age might lead to an increased incidence of neonatal hypoglycemia, thereby obscuring the effect of lifestyle interventions. Our meta-analysis could suggest that pregnant women younger than 30 years might be an appropriate population to observe the improved effect on neonatal hypoglycemia post lifestyle intervention.

Traditionally, screening tests for the diagnosis of GDM are performed at 24–28 weeks of gestation. A prospective cohort study of 4,069 women showed that the increase in fetal growth was not obvious when GDM was diagnosed at 20 weeks of gestation, but it was significantly increased when GDM was diagnosed ≥ 28 weeks (70). In addition, fetal growth in obese women increased when GDM was diagnosed at 20 weeks of gestation (70). This indicated that late diagnosis might miss the opportunity for intervention, especially for a high-risk population. Our findings suggest that lifestyle intervention might be effective for reducing the risk of neonatal hypoglycemia when initiated before 28 weeks of gestation. And this increases the question of the current diagnosis time of GDM. It is necessary to conduct GDM screening in early pregnancy to increase the opportunity of benefiting from early intervention. Therefore, we suggest that the diagnostic criteria of GDM in early pregnancy should be determined reasonably.

An observational epidemiological international multi-ethnic investigation found that intrauterine exposure to higher levels of glucose was associated with childhood obesity and insulin resistance, which was independent of maternal BMI and family history of diabetes (71, 72). A prospective study of patients with GDM found that infants whose mothers had the lowest blood glucose levels before and at birth had the lowest incidence of neonatal hypoglycemia (73). Likewise, our results of meta-regression analysis suggested that the risk of neonatal hypoglycemia post lifestyle intervention was lower in patients with GDM having lower maternal fasting glucose levels at trial entry. Thus, maternal fasting glucose levels at trial entry might be the potential source of heterogeneity among studies.

Our meta-analysis has some limitations. One possible limitation may be related to the different GDM diagnostic criteria used in the included studies, which might lead to the inevitable existence of heterogeneity between studies. In fact, the diagnostic criteria of GDM have been controversial (60). In addition, there were a variety of lifestyle interventions included in this study, and the number of studies reporting each intervention was limited, so we cannot determine which specific intervention is more effective. Furthermore, we could not find out how much the degree of hypoglycemia is worse or improves with a change in lifestyle due to the limitation of data. Lastly, significant heterogeneity among studies was found. According to the result of the meta-regression, the heterogeneity might be attributed to the differences in maternal fasting glucose levels at trial entry.

Conclusion

This current meta-analysis of 18 RCTs demonstrated that lifestyle intervention during pregnancy in women with GDM significantly reduced the risk of neonatal hypoglycemia, especially when subjects were younger than 30 years old, or lifestyle intervention initiated before the third trimester, or with dietary intervention. However, future well-designed large-scale trials are still required to further explore the best pattern of lifestyle intervention and to determine the proper early diagnostic criteria for GDM.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

Y-HW, H-HZ, ZZ, and ZY designed and conducted the research, collected and analyzed the data, and wrote the article. Y-HW, H-HZ, ZZ, ZY ZN, JT, ZY, and SZ helped with the data interpretation, contributed to the discussion, and revised the article. ZZ and ZY had primary responsibility for the final content of the manuscript. All authors participated in critically revising and approving the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor LQ declared a shared parent affiliation with the author ZZ at the time of the review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.962151/full#supplementary-material

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Longitudinal changes of lactopontin (milk osteopontin) in term and preterm human milk

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Background: Lactopontin (LPN) in breast milk, also known as milk osteopontin is thought to play a myriad of important roles in infants when they are immature. The purpose of the present study was to examine the longitudinal changes in LPN concentrations in term and preterm milk, and elucidate the links between maternal characteristics, LPN levels, and child growth in a birth cohort.

Methods: 131 mothers who delivered term, moderate-late preterm (MPT), very preterm (VPT), and extremely preterm (EPT) infants were included, milk samples were collected at 7, 14, 28, and 120 days postpartum. LPN concentration was determined by multiple reaction monitoring (MRM) using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Results: Our results indicated that LPN change over time of VPT (P = 0.024) and EPT (P = 0.003) were significantly different from term milk, although they all gradually decreased with lactation. In terms of LPN-related factors, maternal age was a significant contributor in late mature milk and pre-pregnancy BMI a significant contributor to colostrum and transitional milk. We further investigated relationships between LPN levels and infant weight and our results suggested that high levels of LPN in breast milk might be useful for the catch-up growth of infants.

Conclusion: LPN levels in breast milk are related to maternal factors, and differences in LPN levels may affect the growth of infants. As milk is a critical part in the mother–breastmilk–infant "triad," the association between maternal-infant factors and milk LPN levels warrants further study.

KEYWORDS

osteopontin, lactopontin, breast milk, infant, preterm

Introduction

Human milk provides multiple molecular components to guarantee the best start for an infant at a healthy life (1). Among the many different components (lactose, human milk oligosaccharides, lipids, etc.), proteins are crucial nutritional and bioactive molecular factors in human milk. In recent years, osteopontin (OPN) in milk, also known as lactopontin (LPN) (2, 3), has attracted much attention. OPN was first identified in bone tissue as a bone matrix protein (4, 5). OPN was further discovered in various tissues and cells in two forms: intracellular OPN found in immune cells (6) and secreted OPN present in body fluid (7). LPN is a unique type of OPN as its concentration in human milk, especially in colostrum, was much higher than other types of OPN in other tissues and excretions (8). Moreover, LPN showed a higher degree of posttranslational modifications (PTMs) than other types of OPN (9–11).

The high level of LPN in colostrum indicates that LPN may play some roles when the infants are immature (12). Recent studies have discovered a myriad of biological roles of LPN for the development and health of infants. LPN is capable to stabilize calcium by forming a soluble complex with calcium (13). Due to its integrins and receptors binding properties, LPN shows bioactivities in intestinal development, brain development, and immunological development (14).

Preterm birth is defined with gestational age less than 37 weeks. Globally, approximately 15 million preterm infants are born per year and preterm birth complications are the leading contributors to the deaths of children under 5 years of age (15, 16). Compared to term infants, preterm infants have higher vulnerable body surfaces, and more immature organ systems due to shorter intrauterine time (17). Preterm birth can be subdivided based on gestational age: extremely preterm (EPT, less than 28 weeks); very preterm (VPT, 28–32 weeks) and moderate-late preterm (MPT, 32–37 weeks). The shorter the gestational age is, the worse the developmental status of preterm infants (18). Preterm infants, especially the extremely preterm and very preterm infants, are usually accompanied by immature gut and immune system, and therefore, are prone to short- and long-term health consequences, such as infections, growth concerns, developmental impairments, and other comorbidities (19). As suggested in animal studies, LPN might play a critical role in preterm birth. Supplementary LPN was demonstrated to reduce the rate of diarrhea in preterm pigs (20) and, while it did not affect the incidence of necrotizing enterocolitis, it can reduce the severity (21). LPN was identified in preterm breast milk by bottom-up mass spectrometry-based approach (22, 23). However, to the authors' knowledge, LPN concentration in preterm milk, especially the longitudinal changes among the various preterm subcategories was not reported yet.

The purpose of the present study was to examine the longitudinal changes in LPN concentrations in term, MPT, VPT, and EPT milk, and elucidate the links between maternal characteristics, LPN levels, and child growth in a birth cohort. Milk samples were collected at four-time points covering the lactational stages of colostrum (<7 days postpartum), transitional milk (8–14 days postpartum), early mature milk (15–28 days postpartum) and fully mature milk (> 42 days postpartum). LPN concentration was determined by multiple reaction monitoring (MRM) using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Materials and methods

Study population

This study was conducted between December 2017 and April 2020. Women who gave birth to healthy term or preterm infants were recruited from the Department of Pediatrics, Peking University Third Hospital. Eligible criteria included women aged 18 years and older, and with the intention to breastfeed for at least 4 months postpartum. Women who delivered infants with congenital malformations, genetic diseases or required surgery were excluded. A total of 131 women were included. Participants were further grouped into four, according to their infants' gestational age at birth: extremely preterm (EPT, less than 28 weeks); very preterm (VPT, 28–32 weeks); moderate-late preterm (MPT, 32–37 weeks) and full-term (37–42 weeks) (24). The study flow chart is depicted in **Figure 1**.

Baseline information of both mothers and infants were collected through the electronic medical record system, which included maternal height, weight, age, parity, mode of delivery, and infants gestational age and birthweight. Infant weight was obtained from questionnaires completed by parents at 28 and 120 days postpartum, which was accurate to 0.1 kg. Fenton preterm growth charts (25) were used to calculates Z-scores for weight of preterm infants to 49 weeks and WHO child growth standard for term infants and preterm infants after 50 weeks.

This study was approved by the Peking University Third Hospital Medical Science Research Ethics Committee (ethical approval reference: S2016159). All mothers who participated in this study signed the written informed consent.

Milk collection

Breast milk samples were collected from the mothers at 7, 14, 28, and 120 days after delivery. A trained nurse performed the milk expression during hospitalization. Mothers were trained to collect breast milk at home when discharged. The breast milk samples were collected between 9:00 and 11:00 in the morning before breastfeeding. Mothers are told to wash their hands and clean their breasts before collecting breast milk. After that, they

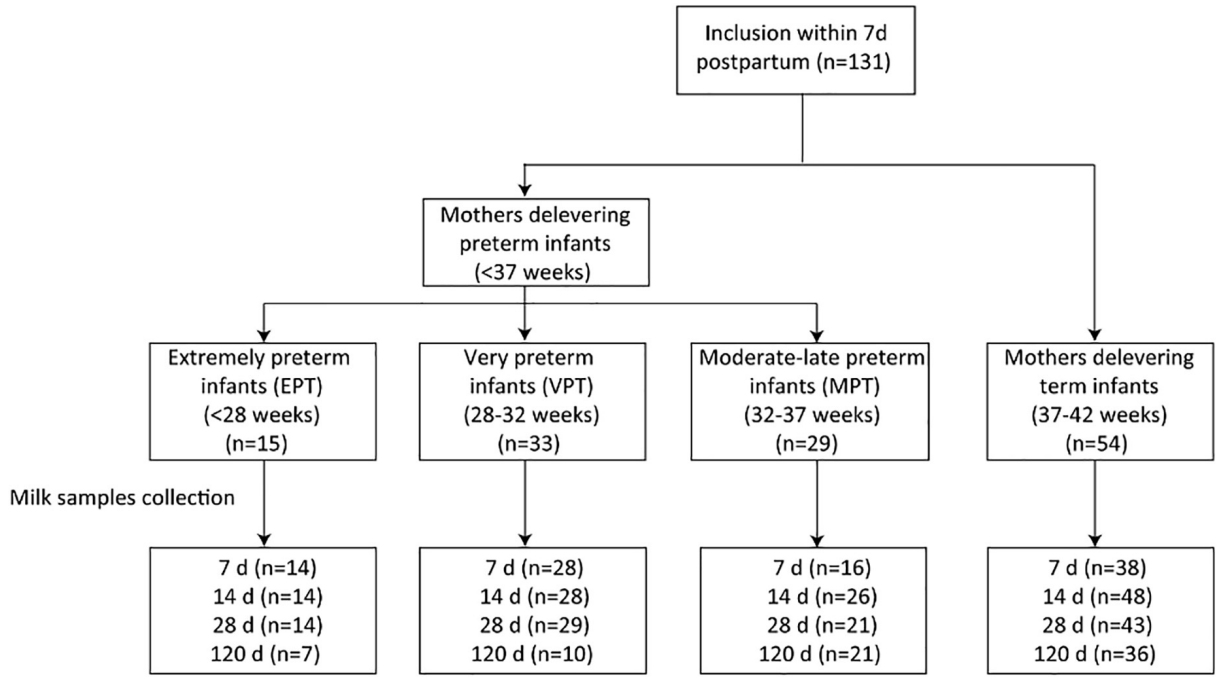

FIGURE 1
Flow chart of the study cohort.

need to manually express or pump full milk expression from a single breast and then homogenized. An aliquot of 10 mL was taken for analysis in collection tubes, and the rest was used to feed the infants. The collected breast milk was temporarily stored in a refrigerator at home and transferred to the hospital as soon as possible within 1–2 days with ice packs, and stored at −80°C until thawed for analysis.

Targeted multiple reaction monitoring analysis for lactopontin

Selection of lactopontin signature peptide

For the targeted MRM analysis, the potential signature peptides were selected by peptides identified in our previous study of bottom-up milk proteome (26). The general rules for targeted proteomics were applied for further selection. In brief, we attempted to avoid peptides which were too short (less than six amino acids) or too long (more than 25 amino acids), contained Met residues or sites for potential modification, and peptides with miscleavege (27). Additionally, the specificity of potential signature peptides was proved by an online BLAST search in NCBI.[1] After exploration, the signature peptide to detect LPN was determined and the sequence was GDSVVYGLR.

1 www.ncbi.nlm.nih.gov

Chemicals and reagents

The signature peptide GDSVVYGLR (purity was 99.01%) and the synthetic isotopic peptide GDSVVYGLR*(purity was 99.63%) were synthesized by GLS Co., Ltd. (Shanghai, China). Analytical grade sodium bicarbonate (NaHCO3) and calcium chloride (CaCl2) were purchased from XILONG SCIENTIFIC Co., Ltd. (Guangzhou, China). Analytical grade acetic acid (HAC) was purchased from BEIJING HUAGONG Co., Ltd. (Beijing, China). HPLC grade dithiothreitol (DTT), iodoacetamide (IAA) were purchased from Sigma-Aldrich (St. Louis, MO, United States). LC-MS grade formic acid (FA) and LC/MS grade acetonitrile (ACN) were purchased from Fisher Chemical (MA, United States). Ultrapure water was generated from a water purification system (Aquaplore, United States). Mass spectrometry grade trypsin was purchased from Promega USA.

Sample preparation

The breast milk samples were thawed at 4°C. Before tryptic digestion, 20 μL milk sample was mixed with 180 μL of 500 mM $NaHCO_3$ and 20 μL 500 mM DTT and the mixture was incubated in a 70°C water bath for 30 min. Alkylation was then performed by adding 60 μL of 500 mM IAA for 30 min at room temperature in the dark. Subsequently, 10 μL of 100 mM $CaCl_2$ and 100 μL of 200 μg/mL trypsin were added and incubated in 37°C water bath for 4 h. The reaction was terminated by the addition of 10 μL of 100% formic acid. After 20 min, 40

μL of 1 μg/mL synthetic isotopic peptide solution and 570 μL of ultrapure water was added to reach a final volume of 1 mL. The mixture was cleaned by a 0.22 μm nylon filter for LC-MS/MS analysis.

Ultra-performance liquid chromatography-tandem mass spectrometry analysis

The UPLC-MS/MS experiments were performed on an ACQUITY UPLC system coupled to TQ-S with electrospray ionization (ESI) (Waters, Milford, MA, United States). The analytical column was Waters XBridge Peptide BEH C18 column (1.7 μm, 2.1 × 100 mm, 300 Å) and the temperature was kept at 35°C. The mobile phase consisted of 0.1% FA aqueous solution (Solvent A) and ACN (Solvent B). The flow rate was 300 μL/min. The gradient consisted of an initial 20%B for 1 min, followed by an increase from 20 to 40%B over 4 min, and followed by a second increase from 40 to 100%B over 0.5 min, the gradient was then decreased to 20%B over 0.5 min and maintained at 20%B for 1 min. The total chromatography time was 7 min and the injection volume was 5 μ L.

The quantitative analysis was performed in MRM mode in duplicates. Mass transitions monitored in the analysis were m/z 483.2 > 345.1 and 483.2 > 607.3 for the signature peptide GDSVVYGLR; and m/z 483.2 > 355.1 and 483.2 > 617.3 for the isotope internal standard peptide GDSVVYGLR*. The synthetic peptide GDSVVYGLR was used to generate the standard curve of intensity vs. concentration. The mass spectrometer parameters were as follows: capillary voltage at 3.0 kV, cone voltage at 40 V, the dissolvent temperature at 350°C, dissolvent gas flow at 700 L/min, tapered hole back blowing gas flow at 150 L/h, collision chamber pressure at 7 bar. More than fifteen points were collected for each peak.

Statistical analysis

Statistical analyses were performed using R statistical software version 4.0.3. Baseline characteristics of the mothers and the infants were presented as mean ± *SD* for continuous variables and frequencies for categorical variables. Continuous variables were analyzed between

TABLE 1 Baseline characteristics of the mothers and infants.

	Term	Preterm				P1	P2
		Total	MPT	VPT	EPT		
Mothers (*n*)	**54**	**77**	**29**	**33**	**15**		
Gestational age at delivery (week)	38.9 ± 1.1	30.8 ± 3.1	34.0 ± 1.0	30.1 ± 1.3	26.1 ± 0.7	<0.001**	<0.001**
Maternal age (year)	33.5 ± 4.2	33.5 ± 4.4	33.3 ± 3.8	33.4 ± 5.2	34.2 ± 3.7	0.926	0.934
Maternal pre-pregnancy BMI (kg/m^2)	22.3 ± 3.2	22.9 ± 3.1	22.8 ± 2.8	22.8 ± 3.3	23.2 ± 3.4	0.293	0.747
Mode of delivery (*n*, %)							
Vaginal	28 (51.9%)	27 (35.1%)	6 (20.7%)	9 (27.3%)	12 (80.0%)	0.055	<0.001**
C-section	26 (48.1%)	50 (64.9%)	23 (79.3%)	24 (72.7%)	3 (20.0%)		
Multiple births (*n*, %)							
Singleton	53 (98.1%)	45 (58.4%)	14 (48.3%)	23 (69.7%)	8 (53.3%)	<0.001**	<0.001**
Twins and triplets	1 (1.9%)	32 (41.6%)	15 (51.7%)	10 (30.3%)	7 (46.7%)		
Maternal parity (*n*, %)							
1	33 (61.1%)	51 (66.2%)	19 (65.5%)	20 (60.6%)	12 (80.0%)	0.547	0.579
> 1	21 (38.9%)	26 (33.8%)	10 (34.5%)	13 (39.4%)	3 (20.0%)		
Mother's education (*n*, %)							
High school or less	0 (0.0%)	11 (14.3%)	3 (10.3%)	6 (18.2%)	2 (13.3%)	0.001**	0.053
Undergraduate	30 (55.6%)	46 (59.7%)	18 (62.1%)	19 (57.6%)	9 (60.0%)		
Postgraduate or above	24 (44.4%)	20 (26.0%)	8 (27.6%)	8 (24.2%)	4 (26.7%)		
Infants (*n*)	55	108	42	44	22		
Sex, boys (*n*, %)	38 (69.1%)	56 (51.9%)	23 (54.8%)	21 (47.7%)	12 (54.5%)	0.035*	0.175
Birthweight (g)	3301.6 ± 453.0	1530.3 ± 540.7	2002.9 ± 416.8	1344.3 ± 339.1	921.6 ± 109.4	<0.001**	<0.001**
Birth length (cm)	49.4 ± 2.0	39.6 ± 4.5	43.2 ± 3.2	38.3 ± 3.6	34.8 ± 2.1	<0.001**	<0.001**
Head circumference (cm)	34.3 ± 1.7	28.5 ± 3.2	31.0 ± 1.8	27.9 ± 2.5	24.3 ± 1.2	<0.001**	<0.001**
Apgar score at 1 min	9.9 ± 0.5	8.9 ± 1.6	9.6 ± 0.8	9.0 ± 1.5	7.4 ± 1.8	<0.001**	<0.001**
Apgar score at 5 min	10.0 ± 0.1	9.5 ± 0.7	9.9 ± 0.3	9.6 ± 0.7	8.7 ± 0.6	<0.001**	<0.001**

P_1, Differences between Term and total preterm conducted by 2-sample t-test; P_2, Differences between the four groups (term, moderate-late preterm, very preterm, and extremely preterm) conducted by ANOVA. Statistical differences were indicated by *P < 0.05, **P < 0.01.

term and preterm using 2-sample *t*-tests, and between four groups (term, moderate-late preterm, very preterm, and extremely preterm milk) using ANOVA tests. Categorical data were analyzed using Pearson χ^2 tests.

To compare the LPN level between three preterm groups and term infants, the term group was taken as reference, and three preterm groups were compared with it separately. To take advantage of the longitudinal design and repeated measurement of LPN, generalized estimating equation (GEE) was used to estimate the main effect and interaction between LPN level and time. If the interaction is significant, the simple effects at each time point were tested using Wilcoxon rank-sum test since the data was not normally distributed, indicated by the Shapiro-Wilk test.

To validate associations between LPN concentrations and maternal characteristics, Pearson correlation was used for continuous variables and Spearman correlation for categorical variables. Pearson correlation was used to validate associations between LPN concentrations and infants' growth, including weight z-scores and weight gain z-scores.

In all statistical tests, results were interpreted as statistically significant where p was less than 0.05.

Results

Study population

The general characteristics of the study population are presented in **Table 1**. In total 131 participants were enrolled in the study. As we divided the participants into 4 groups (term, MPT, VPT, and EPT) based on their gestational ages, it was expected that the gestational ages among these groups were significantly different. The average age of the mother participants was 33.58 ± 4.29 years old, and no significant difference in maternal age was observed among the four groups. The average body mass index (BMI) before pregnancy was 22.50 ± 3.02 and showed no difference among the four groups. As expected, mothers in preterm groups tended to deliver by C-section and give birth to twins and even triplets. Other maternal factors, such as maternal parity, education background, were similar among mothers who delivered term, and preterm babies.

For the infants, the birthweight of term infants were higher than preterm ones, especially the EPT infants only had an average birthweight of 957.69 ± 107.87 g. Other measurements for infants, including birth length, head circumference and Apgar score at 1 and 5 min, were worse for preterm infants. There was no difference in sex distribution.

The lactopontin concentrations change over time in term, moderate-late preterm, very preterm, and extremely preterm milk

As illustrated in **Figure 2** and **Supplementary materials**, for both term and preterm milk (MPT, VPT, and EPT), LPN levels decreased dynamically throughout the lactational periods from colostrum to fully mature milk. The result of GEE indicated that LPN change over time of VPT (P = 0.024) and EPT

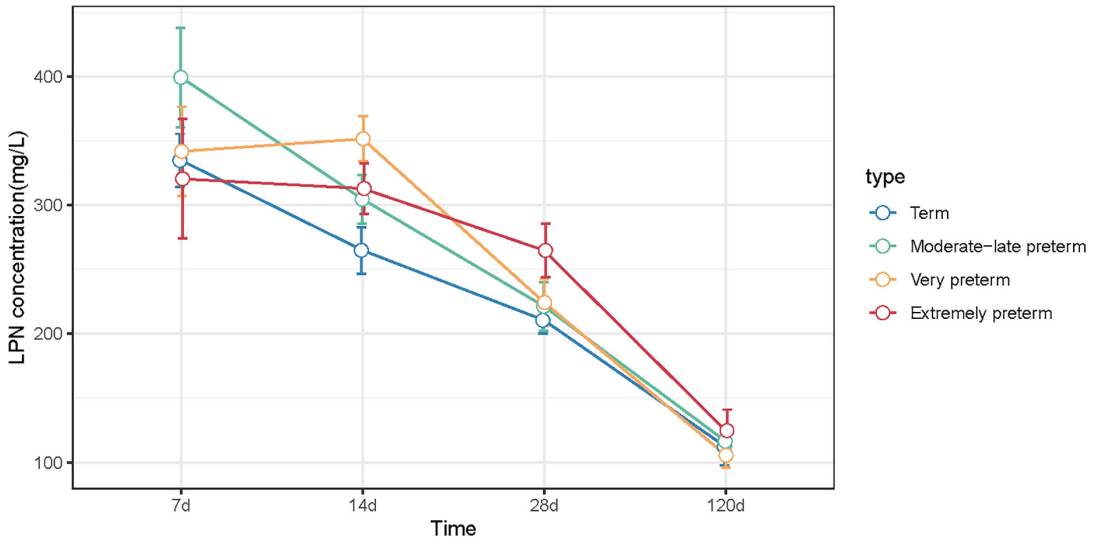

FIGURE 2
LPN concentrations of term, MPT, VPT, and EPT milk at 7, 14, 28, and 120 days postpartum. Statistical differences compared to term group were indicated by *P < 0.05, **P < 0.01.

(P = 0.003) were significantly different from term milk. We further compared the simple effects at each time point. At 14 days postpartum, the level of LPN in VPT milk (medium: 351.48 vs. 264.76 mg/L, P = 0.009) was significantly higher than term milk; at 28 days postpartum, LPN in VPT milk (224.33 vs. 210.45 mg/L, P = 0.038) and EPT milk (264.75 vs. 210.45 mg/L, P = 0.007) was significantly higher than term milk. However, no difference was observed between MPT and term milk. Our result indicated that mothers who delivered most immature infants tended to produce more LPN in early mature milk.

Lactopontin levels in relation to maternal characteristics

The correlation between LPN levels and maternal characteristics was shown in Table 2. Maternal age was positively associated with LPN levels at 120 days postpartum. For the maternal pre-pregnancy BMI (kg/m2), it was positive associated with LPN levels at 7 and 14 days postpartum. No correlation was found between mode of delivery, multiple births, maternal parity, mother's education background, and LPN levels.

TABLE 2 Maternal factors and their correlation with milk LPN levels.

Maternal factors	Lactation (days)	r	P_1	P_2
Maternal age (year)	7	0.183	0.083	–
	14	0.088	0.357	–
	28	0.121	0.223	–
	120	0.273	0.019*	–
Maternal pre-pregnancy BMI (kg/m^2)	7	0.289	0.005**	–
	14	0.201	0.034*	–
	28	0.107	0.288	–
	120	0.216	0.073	–
Mode of delivery	7	0.003	–	0.977
	14	0.109	–	0.245
	28	0.103	–	0.290
	120	0.222	–	0.057
Multiple births	7	0.032	–	0.755
	14	0.108	–	0.247
	28	0.190	–	0.050
	120	0.113	–	0.337
Maternal parity	7	−0.007	–	0.945
	14	−0.123	–	0.189
	28	−0.130	–	0.183
	120	−0.034	–	0.775
Mother's education	7	−0.082	–	0.430
	14	0.067	–	0.474
	28	0.005	–	0.959
	120	0.076	–	0.518

P_1, Pearson correlation test; P_2, Spearman correlation test. Statistical differences were indicated by *P < 0.05, **P < 0.01.

Relationships between lactopontin levels and infant weight

In this study, we investigated the relationship between LPN concentrations and infant weight in all infants, as well as in term and preterm infants (Table 3). In total infants, LPN concentration was negatively associated with infant weight z-score at 28 days postpartum. However, this difference was not significant at 120 days postpartum, which suggested that high levels of LPN in breast milk might be useful for the catch-up growth of infants.

Discussion

This study measured LPN levels in term and preterm milk at four time points covering the four lactational stages of colostrum (< 7 days postpartum), transitional milk (8–14 days postpartum), early mature milk (15–28 days postpartum) and fully mature milk (> 42 days postpartum). To our knowledge, this study provides the first dataset of the longitudinal changes of LPN levels in preterm subcategories.

Currently, most published studies measured LPN levels by enzyme-linked immunosorbent assay (ELISA) (5, 28, 29). ELISA is widely used for biochemical analysis as its high specificity and sensitivity by utilizing the specific binding of antigen to antibody. However, data acquired from different commercially ELISA kits showed variable and non-comparable absolute concentrations (30). Moreover, the ELISA method is of concern since cross-reactivity or non-specific antibody recognition have been noticed in complex sample matrix and lead to false negative or false positive (31). Such problems have been observed in the article (28) mentioned that a study (5) detected LPN levels using an ELISA that was not validated for use in human milk, the detection levels of LPN was shown to overestimate up to 10-fold from the actual levels. Therefore, there is a need for more sensitive and accurate assays allowing for reliable detection of LPN in human milk. Recent studies, including our previous study, used a mass-spectrometry (MS)-based approach to confidently determine the presence of LPN protein (22, 26) or endogenous peptides (23, 32) in human milk, whereas these studies remain semiquantitative at best. Hereby, in this study, we quantified the LPN levels by targeted proteomics in MRM mode which allowed the consistent monitoring of peptides of interest with a high degree of specificity and sensitivity (27).

We found that the median levels of LPN in colostrum were higher than 300 mg/L, and decrease over time, which were similar for the term and the three preterm milk groups (i.e., MPT, VPT, and EPT). The LPN levels were discussed in several studies but the result contrasts. Jiang and Lönnerdal reported during the 1-year lactation period, the highest LPN content in colostrum was 178.0 mg/L, which dropped to 48.3 mg/L at 12 months of lactation (29). Bruun et al. found LPN levels

TABLE 3 Pearson correlation between LPN Levels and infant weight.

	Lactation (days)	$r_{infantweightz-score}$	$P_{infantweightz-score}$	$r_{infantweightgainz-score}$	$P_{infantweightgainz-score}$
Total	28	−0.243	0.041*	−0.071	0.558
	120	−0.060	0.610	0.103	0.380
Term	28	−0.040	0.815	0.298	0.074
	120	0.004	0.981	0.161	0.378
Preterm	28	−0.277	0.113	−0.213	0.234
	120	−0.201	0.197	0.005	0.976

Statistical differences were indicated by *P < 0.05.

varied across countries, from 99.7 mg/L in Danish, 185.0 mg/L in Japanese, 216.2 mg/L in Korean to 266.2 mg/L in Chinese mothers (28). The LPN levels in Chinese mothers' breast milk in our study was in line with Bruun's study.

However, the concentrations of LPN in term and preterm milk showed different trends over time. LPN levels were higher in transitional milk and early mature milk of VPT and EPT group, suggesting it may play important roles in preterm infants. Higher levels of LPN in colostrum and its decreasing trend indicates that LPN may be critical for early infancy (12). A randomized controlled trial of LPN supplementation suggested it may support improved innate and adaptive immune development, in which 240 healthy infants fed either standard formula or formula adding two doses of supplemental LPN or breast milk showed the benefit of LPN supplementation than standard formula in the plasma levels of several amino acids and cytokines, serum levels of the tumor necrosis factor-alpha (TNF-α), and LPN supplementation showed higher similarity to the breastfed infants in many aspects than infants fed standard formula alone (33, 34).

LPN levels might be affected by multiple factors like race and environment (diet, geographical factors, etc.) (28). Even in the same race, milk is known to be a dynamic biofluid reflecting the health status of maternal-infant dyads and forming the bridge between two interconnected individuals (35). Hereby, it is expected that we could notice a remarkable individual variance of milk LPN levels in our study. Our results have also demonstrated maternal age a significant contributor in late mature milk and pre-pregnancy BMI a significant contributor to colostrum and transitional milk. It was interesting that different maternal factors associated with LPN concentrations during specific lactation periods.

As milk is tailored to meet infants' needs and a critical part in the mother–breastmilk–infant "triad" (36), the higher LPN level in these mothers may indicate the higher needs of LPN for their infants. We further investigated relationships between LPN levels and infant weight. Overall, LPN concentration was negatively associated with infant weight z-score at 28 days postpartum and this difference was not significant at 120 days postpartum. Our results suggested that high levels of LPN in breast milk might be useful for the catch-up growth of infants. However, Aksan et al. showed positive correlations between LPN levels and infants' bodyweight and length at first and third months (37). However, in Aksan's study only the LPN concentration in a single collection of breast milk was measured, so the relationship between LPN concentration and infant weight in the results was not derived from longitudinal data.

However, this work has some limitations to consider. First, since the study only investigated 131 women and their infants from one hospital, the results of this study needs to be confirmed in a broader study population. Second, the different sample sizes between 4 groups, and loss of participants during the 4-month follow-up period, limited the power of statistical analysis. Loss of follow-up was more common in the preterm groups than in the term group, especially in extremely preterm group and very preterm group. Therefore, when analyzing the relationship between LPN levels and the growth of infants, we combined the preterm groups. Third, the growth and development of breastfed infants are mainly affected by the total energy in breast milk, so the correlations of LPN levels with infant weight in this study cannot exclude the influence of confounding factors. Additional studies are required to clarify these relationships further.

Conclusion

In summary, we quantified the LPN levels by targeted proteomics in MRM mode with a high degree of specificity and sensitivity. Our results indicated that the concentrations of LPN in term and preterm milk showed different trends over time, although both gradually decreased with lactation. In terms of LPN-related factors, maternal age was a significant contributor in late mature milk and pre-pregnancy BMI a significant contributor to colostrum and transitional milk. We further investigated relationships between LPN levels and infant weight and our results suggested that high levels of LPN in breast milk might be useful for the catch-up growth of infants. As milk is a critical part in the mother–breastmilk–infant "triad," the association between maternal-infant factors and milk LPN levels warrants further study, especially in preterm infants.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the Peking University Third Hospital Medical Science Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

XY and XT: conceptualization and investigation. JZ and SB: methodology. JZ, YX, and YW: formal analysis. JZ and YX: writing—original draft preparation. JL, XY, and XT: writing—review and editing. JZ: visualization. YX and XT: project administration. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.962802/full#supplementary-material

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Concentration and distribution of sialic acid in human milk and its correlation with dietary intake

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Purpose: This study evaluates the content, distribution, and changing trend of sialic acid in human milk and the correlation between dietary intake of sialic acid and that in human milk.

Methods: The study included 33 mothers of full-term and exclusively breastfed infants. At least 2 ml of milk was collected on the 3rd, 8th, 30th, and 90th day after delivery, and 24-h diet recalls of the lactating mothers were obtained each time. The correlation of human milk sialic acid concentration with lactating women's dietary sialic acid intake during lactation was analyzed by statistical analysis software SPSS.

Results: The average concentration of sialic acid in colostrum, transition, and 1 and 3 months were 1,670.74 ± 94.53, 1,272.19 ± 128.74, 541.64 ± 55.2, and 297.65 ± 20.78 mg/L, respectively. The total sialic acid concentration in colostrum was about 5.6 times higher than that at 3 months ($P < 0.001$). The average dietary sialic acid intake of lactating mothers on the 2nd, 7th, 30th, and 90th day after delivery were 106.06 ± 7.51, 127.64 ± 8.61, 120.34 ± 10.21, and 95.40 ± 6.34 mg/day, respectively. The intake of sialic acid was relatively high on the 7th day, and there was no significant difference in dietary intake of sialic acid on different days ($P < 0.05$). In addition, there was no correlation between the intake of dietary sialic acid and the content of total sialic acid and various forms of sialic acid in milk ($P < 0.05$).

Conclusion: During the lactation period, the distribution of sialic acid in breast milk is relatively stable and its content fluctuates greatly, which may not be affected by the mother's diet, but mainly depends on the self-regulation oft physiological needs.

KEYWORDS

human milk, diet, sialic acid, distribution, content

Introduction

Sialic acid is widespread in human tissues, especially the central nervous system. Most of the sialic acid in brain tissue binds to gangliosides, accounting for about 65% of the total sialic acid content of the brain; about 32% is bound to glycoproteins, and the rest exists in the free form (1). The high content of sialic acid is an important material basis of nervous system function, meaning that it may play a unique structural and functional role in the nervous system. Interventions with sialic acid can have long-term irreversible effects on the brain during critical periods of brain development.

Wang (2) fed piglets with sialic acid-supplemented formula, and the eight-arm maze test was conducted to evaluate their learning and memory ability. The results showed that sialic acid affected the brains of piglets and could significantly improve their learning and memory ability. Morgan and Winick's study (3) confirmed that increasing exogenous sialic acid intake could promote ganglioside sialic acid production in the brain of young rats and improve learning ability, and these changes will persist into adulthood. Moreover, some studies had found that *N*-acetylneuraminic acid supplementation to the mother rats during pregnancy could enhance the brain Neu5Ac and promote the cognitive development of the offspring but had no such effect with rats that missed the period of rapid brain development (4, 5). Thus, these findings suggest that sialic acid is vital for brain growth and development, but only increased exogenous sialic acid intake early in life affects its content in brain tissue.

All mammals, including humans, can synthesize endogenous sialic acid. However, endogenous synthesis is limited for newborns because the liver and other organs are not yet matured. Hence, infants need adequate exogenous sialic acid to meet rapid brain development needs. Human milk is the primary exogenous source of sialic acid, which mainly exists in combined form in oligosaccharides, glycoproteins, and glycolipids in human milk. Studies have shown that sialic acid is abundant in colostrum and tends to decrease with prolonged lactation (2, 6). Changes in the content of sialic acid in human milk can be affected by various factors, and whether they are affected by dietary factors is unknown. There is relatively little research on the composition, distribution, and related influencing factors of sialic acid in human milk. We analyzed the distribution, content, and trend of sialic acid in human milk, its changing trend with lactation continuation, the correlation between dietary sialic acid intake and sialic acid in human milk, and discussed its nutritional significance.

Materials and methods

Dietary sialic acid survey

The study included 33 mothers of full-term and exclusively breastfed infants from the Maternal and Child Care Service Center in Xiamen, China. The household survey was conducted on the 3rd, 8th, 30th, and 90th postnatal days, and a 24-h dietary recall of all foods and drinks consumed was obtained on the survey days. We use a questionnaire combined with a special food quantity reference measurer for dietary survey, including commonly used food pictures, quantitative standard molds and common food weight tables to assist respondents in recall, qualitative and quantitative analysis. Record the variety, brand name, texture and processing of food. Fill in in grams, if other units, convert to grams, and accurately calculate the type and weight of food. The investigation was conducted by specially trained investigators using the same protocol. In addition, follow-up data collection for quality control was conducted through home visits or telephone calls to verify and supplement the data. The dietary composition of sialic acid was determined in the laboratory, and the intake of dietary sialic acid in lactating women was estimated. The study was approved by the Medical Ethics Committee (Ethics No: XDYX2020008), and all the mothers gave written informed consent before inclusion in the study.

Basic information of subjects

Almost all the mothers surveyed were the first-born, mainly permanent residents of Xiamen, basically Han nationality, with a good level of education. The average age of all mothers was about 27 years old, and the average height was 160cm. Their body weight decreased with lactation, but the average body mass index (BMI) was within normal range. All the nursing mothers are healthy non-smokers and have no family history of genetic diseases. They don't drink or take drugs in puerperium, and they start to exercise moderately after the month.

Human milk collection

Breast milk samples (2–10 ml) were collected using a breast pump at approximately the same time of the morning (09:00–11:00) on 4 occasions: 3rd (colostrum), 8th (transition milk), 30th (mature milk), and 90th (mature milk) days after birth. All milk samples were stored in clean bottles in a −20°C refrigerator until analyzed.

Preparation and determination of human milk samples

Preparation and handling of human milk samples

First, 500 μl of milk was accurately aspirated in a centrifuge tube, and an equal volume of 10% trichloroacetic acid solution was added to precipitate the protein. The solution was thoroughly mixed, then placed in an ice bath for 10 min, centrifuged at 4°C, 3,000 (×g) for 30 min, and the supernatant was collected. Secondly, 500 μl cold 5% trichloroacetic acid was added to the precipitate, mixed well, centrifuged for 30 min at 4°C, 3,000 rpm, and the supernatant was mixed with the previous one. Next, 500 μl of supernatant was taken in a centrifuge tube and filtered through a 0.22 μm membrane for free sialic acid detection. The remaining supernatant was added with an equal volume of 0.1 mol/L trifluoroacetic acid, hydrolyzed at 80°C for 30 min, and filtered through a 0.22 μm filter membrane after cooling. Taking the filtrate for detection, the sum of free sialic acid and oligosaccharide-bound sialic acid in human milk can be obtained. Subtracting free sialic acid from this part of the result is oligosaccharide-bound sialic acid. In addition, 2 ml (0.05 mol/L) sulfuric acid was added to the precipitated protein, hydrolyzed at 80°C for 120 min. After cooling, it was filtered through a 0.22 μm filter membrane, and the liquid was taken for protein-binding sialic acid detection (7).

Although a small part of sialic acid also exists in the form of glycolipids (gangliosides), its proportion in the total sialic acid content of human milk is very low (<0.5%) (8); therefore, it was not detected separately in this study (included in free sialic acid).

Chromatographic detection conditions

Chromatographic column: waters C18 (2.5 μm, 2.1 × 150 mm), protective column: waters C18 (2.5 μm, 2.1 × 20 mm), column temperature 30°C; The excitation wavelength of the fluorescence detector was 373 nm, and the emission wavelength was 448 nm; The mobile phase was methanol-acetonitrile-ultrapure water (2.5:3.5:94); the flow rate was 0.3 ml/min; the injection volume was 10 μl.

Dimethyl balenine (DMB) derivatization solution: 8 mM DMB, 1.5 M glacial acetic acid, 0.25 M sodium hyposulfifite, 0.25 M sodium sulfifite, and 0.8 mM 2-mercaptoethanol.

Derivatization conditions: 10 μl DMB derivatization solution was added to 90 μl of filtered samples or standards, derivatized at 50°C for 150 min in the dark, cooled to room temperature, and analyzed by liquid spectrometry (4).

Repeatability test: the colostrum of the same person was measured for six repeated samples, 500 μl each. After injection according to the sample treatment method, the peak area was determined to be 7,456, 7,436, 7,320, 7,459, 7,489, and 7468 respectively, and the calculated RSD was 0.8%, indicating that the detection method had good repeatability.

Statistical analysis

The one-way ANOVA in a simple linear model was used to evaluate the variation among the four lactation periods. Bonferroni correction and repeated measures ANOVA were used to analyze the dynamic changes of sialic acid contents of dietary intake and in human milk of nursing mothers during lactation. Shapiro–Wilk test and Spearman's correlation coefficient were used to determine the correlation between sialic acid content in the mothers' dietary intake and the milk secreted. The significance level was set at $P = 0.05$.

Results

Sialic acid content and changing trend in human milk

Table 1 shows that colostrum had the highest (1,670.74 mg/L) sialic acid content. As the lactation period increased, the sialic acid content in human milk decreased rapidly. The content of transition milk decreased to 1,272.19 mg/L, and mature milk content at 1 month and 3 months was 541.64 and 297.65 mg/L, respectively. The total sialic acid concentration in colostrum was about 5.6 times that of mature milk at 3 months ($P < 0.001$), with 16%−19% of that in colostrum. The concentration of total sialic acid in human milk was negatively correlated with postpartum days ($r = 0.834$ $P < 0.001$). The difference in total sialic acid concentration among different time points was statistically significant ($P < 0.05$). This concentration difference was also reflected in oligosaccharide-bound, protein-bound, and free sialic acid at the four-time points during lactation ($P < 0.05$).

The total sialic acid concentration and that in each fraction showed a decreasing trend. After the repeatability test, it was found to change significantly with continued lactation ($F = 200.796$, $P < 0.001$). The concentration of sialic acid in milk differed between species ($F = 174.97$, $P < 0.001$). The changing trend of sialic acid in different forms differed with time ($F = 119.1$, $P < 0.001$), and the decreasing trend of oligosaccharide-bound sialic acid was more obvious than that of protein-bound and free sialic acid.

Distribution of sialic acid in human milk

Most of the sialic acid in human milk (67.6%−76.0%) was bound to oligosaccharides, 20.9%−29.4% combined with protein, and 2.2%−3.6% was free sialic acid. Figure 1 shows that no matter the lactation stage, the proportion was highest in oligosaccharide-bound sialic acid, and the ratio of the three components was unchanged. As shown in Figure 2, the contents of free, oligosaccharide-bound, protein-bound, and total sialic

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TABLE 1 The concentration of sialic acid in human milk at 4-time points during lactation (mg/L, mean ± SE, $n = 33$).

Distribution of sialic acid	Colostrum	Transition	Mature, 30th day	Mature, 90th day
Free SA	51.58 ± 2.92	38.08 ± 3.85*	16.52 ± 1.78*	9.26 ± 0.60*
Oligosaccharide-bound SA	1,210.71 ± 68.13	917.28 ± 92.40*	395.71 ± 40.75*	213.57 ± 15.23*
Protein-bound SA	408.45 ± 25.16	316.82 ± 33.28*	129.41 ± 12.89*	74.82 ± 5.13*
Total SA	1,670.74 ± 94.53	1,272.19 ± 128.74*	541.64 ± 55.20*	297.65 ± 20.78*

*Indicates that there is a statistical difference compared with the previous time point, $P < 0.05$.

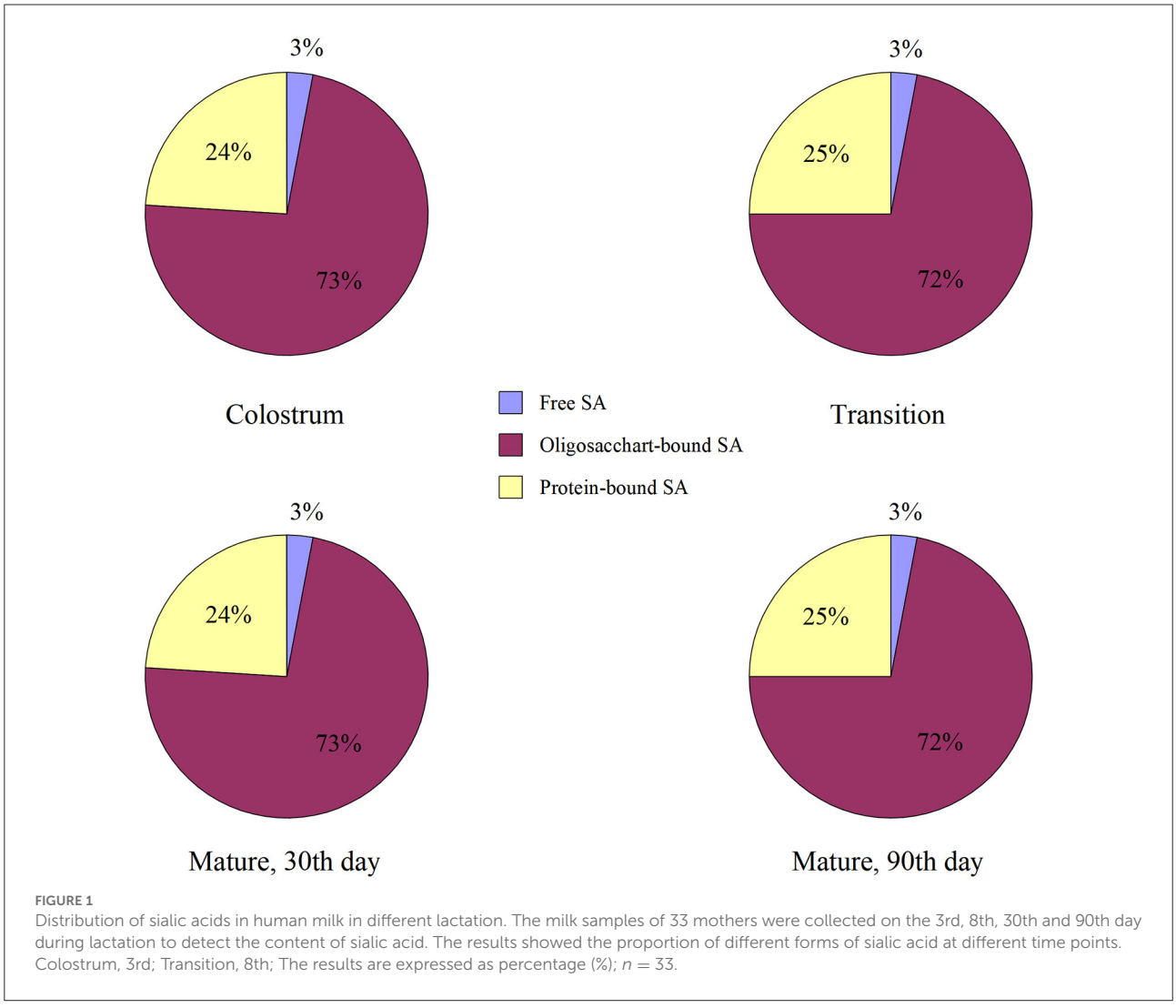

FIGURE 1
Distribution of sialic acids in human milk in different lactation. The milk samples of 33 mothers were collected on the 3rd, 8th, 30th and 90th day during lactation to detect the content of sialic acid. The results showed the proportion of different forms of sialic acid at different time points. Colostrum, 3rd; Transition, 8th; The results are expressed as percentage (%); $n = 33$.

acid in the colostrum are used as references. The content of total sialic acid in transitional milk, one-month mature milk, and 3-month mature milk was 81.41%, 32.99%, and 19.98% of colostrum; the corresponding content of free sialic acid was 79.59%, 32.42%, and 20.21%; the content bound with oligosaccharides was 80.59%, 33.20% and 19.69%; and that bound to protein was 84.78%, 32.84%, and 20.99%. With the prolonged lactation period, the total sialic acid and that in other forms decreased significantly compared with that in the colostrum, and the proportion of decrease was similar.

Correlation analysis between dietary sialic acid intake and sialic acid content in milk

Table 2 shows the average dietary Neu5Ac intake of lactating mothers on the 2nd, 7th, 30th, and 90th days after delivery. The average dietary intake of total sialic acid of lactating mothers was the highest on the 7th day after delivery (127.6 ± 48.61 mg/day) and decreased by about 25% in the 3rd month ($P < 0.05$).

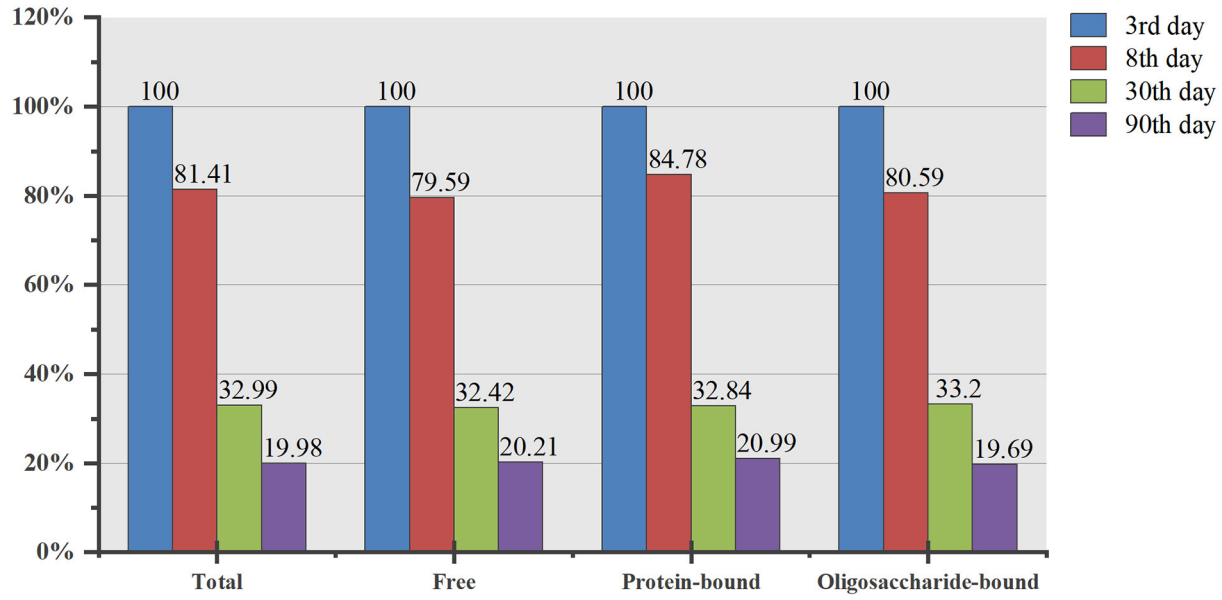

FIGURE 2
Variation trend of total and each fraction of sialic acid compared with colostrum. The results are expressed as percentage (%); $n = 33$.

TABLE 2 The concentration of sialic acid in human milk at 4-time points during lactation (mg/L, mean ± SE, $n = 33$).

	Dietary total SA intake (mg/day)	Total SA concentration in milk (mg/L)	Total SA content in milk (mg/day)	Oligosaccharide-bound SA content in milk (mg/L)	Protein-bound SA content in milk (mg/L)	Free SA content in milk in milk (mg/L)
2nd day	106.06 ± 7.51	1,670.74 ± 94.53	167.07 ± 9.45	1,210.71 ± 68.13	408.45 ± 25.16	51.58 ± 2.92
r^*		0.127	0.127	0.102	0.186	0.135
P-value		0.480	0.480	0.574	0.300	0.453
7th day	127.64 ± 8.61[a]	1,272.19 ± 128.74	636.09 ± 64.37	917.28 ± 92.4	316.82 ± 33.28	38.08 ± 3.85
r^*		0.152	0.152	0.152	0.143	0.141
P-value		0.398	0.398	0.399	0.427	0.435
30th day	120.34 ± 10.21[a]	541.64 ± 55.2	352.07 ± 35.88	395.71 ± 40.75	129.41 ± 12.89	16.52 ± 1.78
r^*		−0.280	−0.280	−0.287	−0.244	−0.261
P-value		0.114	0.114	0.105	0.171	0.143
90th day	95.40 ± 6.34	297.65 ± 20.78	267.89 ± 18.7	213.57 ± 15.23	74.82 ± 5.13	9.26 ± 0.6
r^*		0.135	0.135	0.138	0.120	0.132
P-value		0.455	0.455	0.445	0.506	0.464

*Spearman correlation coefficient between dietary sialic acid intake and sialic acid content and concentration in human milk.
[a]$P < 0.05$, compared with 90th Dietary Total SA intake.

The average milk yield of lactating mothers on the 3rd, 8th, 30th, and 90th day after delivery was 100, 500, 650, and 900 ml, respectively, while the total amount of sialic acid secreted by lactating mothers was 167.07 ± 9.45, 636.09 ± 64.37, 352.07 ± 35.88, and 267.89 ± 18.7 mg, respectively. And the result was estimated according to the average milk yield and the concentration of sialic acid in milk. The amount of sialic acid consumed from the diet on the 2nd, 7th, 30th and 90th days was 106.06 ± 7.51, 127.64 ± 8.61, 120.34 ± 10.21, and 95.40 ± 6.34 mg, respectively. Statistical analysis showed that there were significant differences in dietary sialic acid intake among lactating mothers at different time points, and the dietary sialic acid intake on the 7th day and 30th day was significantly higher than that on the 90th day ($P < 0.05$). In addition, the total amount of sialic acid in breast milk at different postpartum time points was higher than that of dietary intake.

The results of the normality test of dietary sialic acid intake of lactating mothers and sialic acid content in milk at four different time points showed that the data were normally distributed ($P > 0.05$), while the data of total sialic acid secretion and content in each milk form were not normally distributed ($P < 0.05$). Therefore, Spearman correlation was used to analyze the correlation between dietary sialic acid and that in milk; no significant correlation was observed ($P > 0.05$). Similarly, there was no statistically significant correlation between the total and other forms of sialic acid and the dietary sialic acid intake ($P > 0.05$) during different lactation periods.

Discussion

In this study, the sialic acid content in human milk was inversely correlated with postpartum days and gradually decreased with the prolonged lactation period. There was a great difference in the concentration at different times. This trend of sialic acid content in milk results from long-term human evolution, which is adapted to the characteristics of infant growth and development. Compared with other foods, the sialic acid content in human milk is very high (9), especially in colostrum. Sialic acid is mainly synthesized in the liver; GNE (UDP-GlcNAc 2-epimerase) is a key enzyme in regulating sialic acid biosynthesis (10). In the neonatal period, this enzyme cannot meet the needs of rapid brain development due to the immature liver and limited ability to regulate sialic acid synthesis. However, the high sialic acid content in early colostrum can be used as an exogenous supplement to make up for the deficiency of endogenous synthesis in newborns. With continued lactation, the liver function of infants gradually improves, and the ability to synthesize sialic acid gradually improves, which can meet the baby's needs to a greater extent and reduces exogenous dependence. At this time, the decreasing trend of sialic acid content in milk conforms to the changes in the baby's needs. Therefore, the different sialic acid requirements of babies in different lactation periods may lead to different levels of sialic acid in milk, and the dynamic changes of sialic acid in human milk may be determined by different lactation needs.

In addition to the fluctuation of sialic acid content with time, there are also great differences among different individuals, confirmed by many earlier studies (11, 12). In this study, the total sialic acid content of different individuals fluctuated greatly at four different lactation stages. The maximum sialic acid content of 1-month mature milk is about 6.8 times the minimum. The lipid content of human milk is the most variable, and the lipid content of different individuals varies by about 7 times (0.4–2.8 g/100 ml) in 1-month mature milk (13, 14), and the content of sialic acid varies among individuals. Thus, sialic acid is one of the most obvious variable components of human milk. The reason for variation in the sialic acid content of human milk of different individuals is unclear. It may be due to genetic differences that cause different sialic acid synthesis capabilities; that is, the enzymatic activity of synthesizing sialic acid in different lactating mothers is different, and the amount of synthesized sialic acid may also be different. It is also possible that the stimulation of environmental factors leads to different utilization rates of sialic acid *in vivo*.

From the existing form of sialic acid in human milk, it is mainly in the form of oligosaccharide-bound. The sialic acid concentration in all fractions decreased significantly with a longer lactation duration. However, the proportion of sialic acid composition in each fraction did not differ significantly between lactation periods. Oligosaccharide-bound sialic acid always occupies a high proportion in human milk, which may be determined by the content of oligosaccharides in human milk. The main sialylated oligosaccharides in human milk were 3′-SL (3′-siaiyllyatose) and 6′-SL (6′-siaiyllyatose). The concentration of 6′-SL was the highest in colostrum (250–1,300 mg/L) and then decreased gradually, while the concentration of 3′-SL was relatively stable (76–300 mg/L) (15). Studies have shown that human milk contains an appreciable amount of oligosaccharides, with an average content of 12.9 g/L (16), the third-largest component of human milk solids. There are no less than 200 kinds of oligosaccharides in human milk (17). This creates good conditions for combining sialic acid and oligosaccharides in human milk. Oligosaccharides in human milk can not only resist some enterovirus and bacterial infections (18) and participate in the development of a healthy intestinal environment but also stabilize sialic acid in milk and avoid its related enzymatic hydrolysis, and even alter the sialic acid metabolism pathway to improve the half life. According to the above, oligosaccharides are very important components in breast milk. After all kinds of oligosaccharides are sialylated, they endow breast milk with unique functional advantages, which are not available in ordinary formula milk and cow milk. In this study, we determined the total concentration of all kinds of oligosaccharide-bound sialic acid after hydrolysis, without subdividing specific sialic acid containing oligosaccharides. Further detection of the type and concentration of oligosaccharide-bound sialic acid may be more specific to show the distribution of sialic acid in breast milk and its relationship with diet.

During lactation, especially within 1 month after delivery, mothers pay more attention to various nutrients intake; thus, food intake is adequate, and the food structure is reasonable. The effects of the dietary intake of lactating mothers on the composition of human milk are complex and diverse, and different nutrients may be affected differently. Studies have shown (19, 20) that the content of macronutrients in human milk is almost not affected by the intake of macronutrients in the maternal diet. However, specific fatty acids that form lipid parts are susceptible to being affected by lactating mothers' diets (21).

The correlation of Neu5Ac concentration in human milk with the dietary intake of lactating women was also not found in

our current study. Besides, there were no significant correlations between the contents of different forms of sialic acid in human milk and the dietary intake of lactating women ($P > 0.05$). This suggests that the content of sialic acid in milk may not be affected by the mother's diet. Human milk nutrients generally come from three sources: some may be synthesized by breast cells, some from the mother's diet, and some from the mother's nutritional reserves (22).

From the previous results, the amount of sialic acid secreted by mothers through milk at different stages of lactation is higher than their dietary intake, especially in the transition milk, where the total sialic acid secretion in milk is about five times higher than the dietary intake. It can be inferred that the sialic acid in human milk mainly comes from self-synthesis or reserve. When the total content of non-dietary sialic acid is significantly higher than dietary intake, the correlation between sialic acid content in human milk and dietary sialic acid intake may appear weak or directly covered.

In addition, the amount of sialic acid taken by lactating mothers through their diet at different times has little change. However, the content of sialic acid in milk shows a downward trend with the prolonged lactation period, which shows that sialic acid in milk may depend more on the physiological needs of lactating mothers than on their dietary intake. Certain self-regulatory mechanisms in the lactating mother's body might be responsible, and dietary sialic acid intake fluctuations are buffered by this mechanism, thus maintaining a relatively balanced physiological state of sialic acid content in milk, which does not change with dietary sialic acid intake. Perhaps when the content of sialic acid in human milk is low or relatively deficient, dietary sialic acid intake might play a supplementary role, and the correlation between dietary and sialic acid in human milk can be seen more clearly. When the sialic acid content in human milk has been at a high level in the corresponding stage, in a plateau phase, excessive dietary sialic acid intake does not affect the overall level of sialic acid in human milk; therefore, there is no significant correlation between diet and sialic acid in milk. The dietary investigation in this study was 24 h before milk collection; this may not rapidly affect the change of sialic acid content in human milk. Dietary intake of sialic acid was mainly in bound form, and the dietary bound form of sialic acid is better absorbed and utilized by body tissues. However, absorption into the blood and secretion into human milk may take time.

Finally, the study considered that the analysis of intake vs. secretion compared to intake vs. concentration, which may better reveal the correlation. Therefore, we not only explored the relationship between dietary sialic acid intake and secretory concentration, but also further analyzed the correlation between total sialic acid intake and secretion. Since the amount of milk secreted by an individual is uneven and fluctuates greatly, it is difficult to get a stable value of milk production. So we take the average value of standard milk from a large sample as a reference to calculate the total amount and make a relevant analysis. However, even when the amount of milk at different stages was taken into account, it failed to further suggest a correlation between diet and sialic acid in milk, but rather verified to some extent that the two were not correlated.

This study draws the following conclusions: The total sialic acid concentrations were highest in colostrum and decreased with the lactation period; Sialic acid in human milk is mostly oligosaccharide-bound, some protein-bound, and a little in the free form. The distribution of sialic acid in human milk is unchanged at different stages of lactation. In addition, the sialic acid in human milk is higher than dietary intake, without any correlation between them; the content in human milk mainly depends on the self-regulation of physiological needs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

QX and YX: conceptualization, software, and writing—original draft preparation. XW, JH, HL, MZ, and YZ: investigation. XC, DG, and HL: resources and supervision. QX, YX, and WZ: data curation. QX: writing review and editing. XC, DG, XW, and HL: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Profiles of total and sn-2 fatty acid of human mature milk and their correlated factors: A cross-sectional study in China

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Fatty acid (FA) in breast milk is beneficial to the growth and neurodevelopment of infants. However, the structure profiles of breast milk FAs and the influencing factors which are crucial for normal function have not been fully elucidated. This study aimed to characterize the profiles of total and sn-2 FAs in human mature milk based on two representative urban areas in China and explore potential sociodemographic determinants. Mothers (n = 70) at 40–100 d postpartum from Beijing and Danyang were recruited according to unified inclusion and exclusion criteria. Total and sn-2 FA compositions were examined by gas chromatography and quantified. Using the Spearman correlation and multiple regression model, we found that the location and maternal education level were the most conspicuous correlated factor. The milk of mothers from Beijing had higher levels of the n-6 series of long-chain polyunsaturated fatty acids (LCPUFA) (C20:2, C20:3n-6, C20:4n-6, n-6PUFA/n-3PUFA, LA/ALA, and ARA/DHA) than that of Danyang, while the opposite was observed in the n-3 series of LCPUFA (C18:3n-3 and Total n-3PUFA). Compared to the milk of mothers with a high school degree or below, those with a bachelor's degree or above had lower SFAs (C10:0, C12:0, C14:0, and Total SFA), n-3 series of LCPUFA (C18:3n-3 and Total n-3PUFA), C18:1n-9t, and higher n-6 series of LCPUFA (C18:2n-6c, C20:2, C20:4n-6, Total n-6PUFA, and n-6PUFA/n-3PUFA). Maternal age, infant gender, pre-conception body mass index (BMI), parity, delivery mode, and gestational weight gain were also associated with total FAs. However, fewer associations were found between the above factors and sn-2 FAs. This study will promote an understanding of human breast milk's lipid profile and help develop a formula more suitable for infants.

KEYWORDS

breast milk, mature milk, total fatty acid, sn-2 fatty acid, sociodemographic factors

Introduction

Human milk is the optimal food for infants during the first 6 months of life. It provides adequate nutrients and numerous bioactive ingredients such as water, carbohydrate, fat, protein, minerals, vitamins, immunoglobulin, and lactoferrin (1–3). However, the composition of human milk differs significantly between and within mothers. Human milk fatty acids (FAs) are the most variable macronutrient (4). The lipid content of human milk varies between 3 and 5% mostly (wider variation also has been reported) (5); it is the major energy source in breast milk and provides 40–50% of an infant's daily energy requirement (6–8). Triacylglycerol (TAG) is the major compound of lipid in breast milk. The property of TAGs mainly depends on the composition and specific position distribution of FAs. Studies have found that saturated fatty acid (SFA), especially esterified palmitic acid (PA; C16:0), preferentially occupies the sn-2 position in human milk (9, 10), while in formulas, PA is mainly located at the sn-1,3 positions (11, 12). A high level of PA at the sn-2 position is reported to promote the absorption of fat and calcium in infants (13), reduce insoluble calcium soap in feces (14), and aid in intestinal microbiota development of infants (15). However, the FA composition in human milk is easily influenced by maternal characteristics (dietary habits (3), lactation, and gestational age (16), duration of pregnancy (12), the stage of lactation (9, 17), and body mass index (BMI) (18, 19) and also external factors such as maternal geographic location (3, 17) and socioeconomic status (2)).

A number of studies have investigated the impact of maternal dietary habits and lactation stage on FA composition in mother's milk (3, 8, 20, 21). Nevertheless, the investigation of profiles of sn-2 FAs in human mature milk is limited, with only a few reported data for the Chinese population (10, 22, 23). Meanwhile, the exploration of influencing sociodemographic factors of sn-2 FAs is still rarely reported.

In this study, we assessed the total and sn-2 FA profiles of mature milk from mothers living in Beijing and Danyang, China. The associations between FA composition and sociodemographic factors (maternal age, BMI before pregnancy, gestational weight gain, maternal education level, parity, infant gender, and region) were explored to elucidate the main characteristics of total and sn-2 FA profiles in Chinese mature milk and to explore the potential factors influencing their composition.

Materials and methods

Subjects

From June to October 2018, women with singleton pregnancies and no diabetes, hypertension, and other chronic diseases were recruited during their obstetrician visits in Beijing (Beijing Maternity Hospital affiliated to the Capital Medical University) and Danyang (Danyang People's Hospital), China (Characteristics between the two study sites were shown in Table A1). These subjects were considered eligible if their infants were delivered at full term and breastfed. For this study, we excluded mothers with the following criteria: birth weight of infants $< 2{,}500$ g or $> 4{,}000$ g; with mental health disorders; unable to answer questions and poor postpartum mood; participated in any nutrition or drug intervention research; took hormones and antibiotics recently; and tobacco use.

Sample size

A sample size of 64 subjects was estimated by the G*Power 3.1.9 software with a significance level of 5%, power of 80%, and expected effect size of 0.3, considering the correlation between total and sn-2 fatty acid of human mature milk and their correlated factors by correlation test. Evaluated by the G*Power 3.1.9 software, the statistical power was 83% with a sample size of 70, a significance level of 5%, and an expected effect size of 0.3.

Ethical and legal considerations

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All the procedures involving human subjects were approved by the Chinese Clinical Trial Registry with the registration number ChiCTR1800018766 (http://www.chictr.org.cn/listbycreater.aspx). Written informed consent was obtained from all subjects.

Collection of information

Data on height, weight (pre-conception weight and gestational weight gain), age, parity, delivery mode, and infant gender were obtained *via* a questionnaire during sample collection. Information such as the mothers' physical condition and lifestyle aspects were also included in the questionnaire.

Human milk collection

On the day when mothers were scheduled for an obstetrical examination, 40–100 days post-delivery, they were asked to breastfeed at 6–8 am and then use an electric breast pump to empty the milk from one of their breasts at home. When they went to the hospital later (9–11 am), the milk from the breast that was previously emptied was collected by an electric breast pump with the help of sampling persons. The milk was mixed and poured into centrifuge tubes which were later wrapped with tin foil and stored at -80°C for further analysis.

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Lipid extraction, measurement of total and sn-2 FAs

Total lipids were extracted from human milk by a revised Mojonnier method (24). The extracted lipids were saponified and the FA methyl esters were obtained by FA methylation, and then analyzed by gas chromatography (GC). Sn-2 monoglyceride (MAG) was hydrolyzed from triglyceride (TAG) and then analyzed by GC following the method by Sahin et al. (25) (see Supplementary material 1, which demonstrates detailed experimental steps).

Statistical analysis

The contents of each FA and sn-2 FA were expressed as mean ± SD and range (minimum∼maximum). All the data were tested for normal distribution using SPSS (Version 20) before analysis. The Spearman correlations between differences in the contents of the FA/sn-2 FA vs. differences in characteristics of mothers and infants (age, infant gender, pre-conception BMI, gestational weight gain, delivery mode, parity, maternal education level, and sampling site) were calculated. The multiple regression model was adopted to estimate the importance of the differences in characteristics of mothers and infants in explaining the dissimilarities in FA/sn-2 FA. All the statistical analyses were performed in the R (Version 4.1.2), using "psych" (26), "reshape2" (27), "relaimpo" (28), and "packfor" (29) packages. Statistical significance was set at a $P < 0.05$. Plots were generated by the "ggplot2" (30) package in R.

Results

Among the 100 screened healthy volunteers, 25 were excluded for not providing complete questionnaire information and five subjects withdrew from the study. A total of 70 mature milk samples were obtained from 70 mothers. The main characteristics of the participants are described in Table 1.

TABLE 1 Characteristics of mothers and infants included.

Characteristics	Mean	Standard deviation
Age (years)	29.61	3.98
Pre-conception BMI (kg/m^2)	21.04	2.66
Gestational weight gain (kg)	13.43	3.94
	Number	**Frequency**
Region		
Beijing	34	48.57%
Danyang	36	51.43%
Delivery mode		
Natural	33	47.14%
Cesarean	37	52.86%
Parity		
1	52	74.29%
>1	18	25.71%
Infant gender		
Male	34	48.57%
Female	36	51.43%
Maternal education level		
Bachelor degree or above	39	55.71%
College degree	12	17.14%
High school degree or below	19	27.14%

Fat content and total FAs composition

As displayed in Table 2, the total lipid content varied amongst subjects, ranging from 1.33 to 7.25 g/100 g (mean: 3.47±1.52 g/100 g). In total, 34 FAs were detected and only 23 FAs whose levels of more than 0.1% (total fatty acids) were shown in Table 2. C4:0, C6:0, C11:0, C13:0, C21:0, C22:0, C24:0, C14:1, C24:1, C22:2, and C20:5*n*-3 were also detected, but the levels were less than 0.1%. In general, monounsaturated fatty acid (MUFA) was the predominant FA (37.57 ± 3.82%), in which C18:ln-9c was found to make up the largest proportion (34.50 ± 3.44%). SFA was the second most abundant FA (34.50 ±3.44%), the largest component of which was C16:0 (20.06 ± 2.20%). The proportion of polyunsaturated fatty acids (PUFA) was the least (27.94±4.36%), among which C18:2n-6c accounted for more than 85% (24.08 ± 4.42%).

Sociodemographic determinants of total FAs

Some potential correlated factors of human milk FAs, namely, pre-conception BMI, parity, maternal education level, location, infant gender, gestational weight gain, delivery mode, and maternal age were individually explored using the Spearman correlation analysis and later tested using the multiple regression model (Figure 1).

A significant correlation was found between maternal education level and FAs (C10:0, C12:0, C14:0, Total SFA, C18:1n-9t, C18:2n-6c, C20:2, C20:4n-6, Total n-6PUFA, C18:3n-3, Total n-3PUFA, n-6PUFA/n-3PUFA, and LA/ALA), and location and FAs (C8:0, C10:0, C12:0, C16:0, C17:0, C20:0, Total SFA, C17:1, C18:1n-9t, C20:1, C22:1n-9, C20:2,

TABLE 2 Total lipid content (g/100 g milk) and fatty acid (% total fatty acids) levels in mature milk[a].

Lipids	All samples ($n = 70$)	
	Mean±SD	Range
Total lipid g/100 g	3.47 ± 1.52	1.33∼7.25
Total SFA, %	34.49 ± 3.88	25.98∼44.53
C8:0	0.11 ± 0.05	0.03∼0.22
C10:0	0.97 ± 0.33	0.29∼1.77
C12:0	3.66 ± 1.43	0.77∼7.28
C14:0	3.38 ± 1.34	1.32∼7.34
C15:0	0.13 ± 0.05	0.07∼0.34
C16:0	20.06 ± 2.20	14.76∼25.14
C17:0	0.22 ± 0.04	0.15∼0.43
C18:0	5.56 ± 1.01	3.77∼8.28
C20:0	0.19 ± 0.08	0.10∼0.45
Total MUFA, %	37.57 ± 3.82	29.15∼49.43
C16:1	1.91 ± 0.46	1.03∼3.18
C17:1	0.18 ± 0.03	0.09∼0.23
C18:1n-9t	0.17 ± 0.20	0.01∼1.45
C18:1n-9c	34.50 ± 3.44	26.63∼44.33
C20:1	0.61 ± 0.30	0.27∼1.89
C22:1n-9	0.20 ± 0.28	0.03∼1.46
Total PUFA, %	27.94 ± 4.36	19.90∼41.62
C18t[b]	0.61 ± 0.29	0.27∼2.07
C18:2n-6c (LA)	24.08 ± 4.42	15.37∼39.21
C18:3n-6	0.14 ± 0.06	0.05∼0.27
C20:2	0.45 ± 0.12	0.29∼1.17
C20:3n-6	0.40 ± 0.14	0.18∼0.88
C20:4n-6 (ARA)	0.57 ± 0.14	0.32∼1.17
Total n-6PUFA	25.70 ± 4.42	17.04∼40.5
C18:3n-3 (ALA)	1.68 ± 0.73	0.47∼3.29
C22:6n-3 (DHA)	0.31 ± 0.15	0.11∼0.96
Total n-3PUFA, %	2.05 ± 0.76	0.86∼3.64
n-6PUFA/n-3PUFA	14.60 ± 6.69	5.46∼36.15
LA/ALA	17.89 ± 9.67	5.87∼42.11
ARA/DHA	2.14 ± 0.76	0.72∼4.17

[a]The fatty acids with average content > 0.1% are shown in the table.
[b]C18t is the sum of C18:1t, C18:2t and C18:3t, representing the level of trans FAs.
LA, linoleic acid; ARA, arachidonic acid; ALA, α-linolenic acid; DHA, Docosahexaenoic acid.

C20:3n-6, C20:4n-6, C18:3n-3, Total n-3PUFA, n-6PUFA/n-3PUFA, and LA/ALA). The milk of mothers from Danyang had higher levels of C8:0, C10:0, C12:0, C17:1, C18:1n-9t, C20:1, C22:1n-9, C18:3n-3, and total n-3PUFA, and lower levels of C16:0, C17:0, C20:0, C20:2, C20:3n-6, C20:4n-6 and lower n-6PUFA/n-3PUFA, LA/ALA, and ARA/DHA ratios. Compared to the milk of mothers with a high school degree or below, those with a bachelor's degree or above had lower C10:0, C12:0, C14:0, Total SFA, C18:1n-9t, C18:3n-3, Total n-3PUFA, and higher C18:2n-6c, C20:2, C20:4n-6, Total n-6PUFA, and n-6PUFA/n-3PUFA, and LA/ALA ratios.

Several associations were observed between age and some SFAs (C8:0, C15:0, C16:0, C17:0, C18:0, and C20:0), delivery mode, and C10:0, Total SFA, C18:2n-6C, Total n-6PUFA, and Total PUFA. For pre-conception BMI, parity, infant gender, and gestational weight gain, there are few correlations shown between these characteristics and FAs.

For C8:0, LA/ALA, n-6PUFA/ n-3PUFA, and total n-3PUFA, these factors can explain more than 30% of the variance in mature milk FAs.

The composition of sn-2 FAs

In this study, 26 sn-2 FAs were examined. In total, 8 major sn-2 FAs were detected in mature breast milk (Table 3), namely, C16:0, C18:2n-6, C18:1, C14:0, C12:0, C18:0, C16:1, and C18:3n-3. These FAs collectively accounted for 92.43% (mean) of sn-2 FAs. Different from the total FAs composition, total SFA (mean: 67.74 ± 5.21%) was predominant in the sn-2 position, followed by MUFA (16.26 ± 2.54%) and PUFA (16.00 ± 3.87%). Notably, C16:0 accounted for more than half of the total sn-2 FAs (50.18 ± 4.74%). Furthermore, approximately 80% of the total C16:0 was in the sn-2 position (79.72 ± 4.29%). C14:0 and C15:0 were also mainly found in the sn-2 position; the average relative molar percentages at the sn-2 position were 66.80 and 75.31%, respectively. However, most MUFAs and PUFAs were located at sn-1, 3 positions.

Sociodemographic determinants of sn-2 FAs

As shown in Figure 2, no associations with statistical significance were observed between sn-2 FAs and parity. Similar to FAs, maternal education level and location were strong predictors for differences in the constitution of mature milk sn-2 FAs. Mothers from Beijing had lower C20:1n-9, C18:3n-3, and C18:3n-6 and higher percentages of C10:0, C14:1, C16:1, C17:1, C18:2t, and C20:2n-6. Mothers with a bachelor's degree or above had higher C14:1, C16:1, C17:1, C18:2t, C18:2n-6, C20:2n-6, and lower C18:3n-3 compared to the mothers with a high school degree or below. Several correlations were also found between age and C18:0, C20:0, and C20:2n-6; delivery mode and C16:0, C17:1 and C18:2n-6; gestational weight gain and C18:0, C17:1; infant gender and C14:1, C18:1, C20:1n-9; pre-conception BMI and C16:1, C18:3n-6.

These factors explained 1.67–35.76% of the variance in sn-2 FAs, with the lowest proportion (1.67%) for C18:3t and the highest proportion (35.76%) for C16:1.

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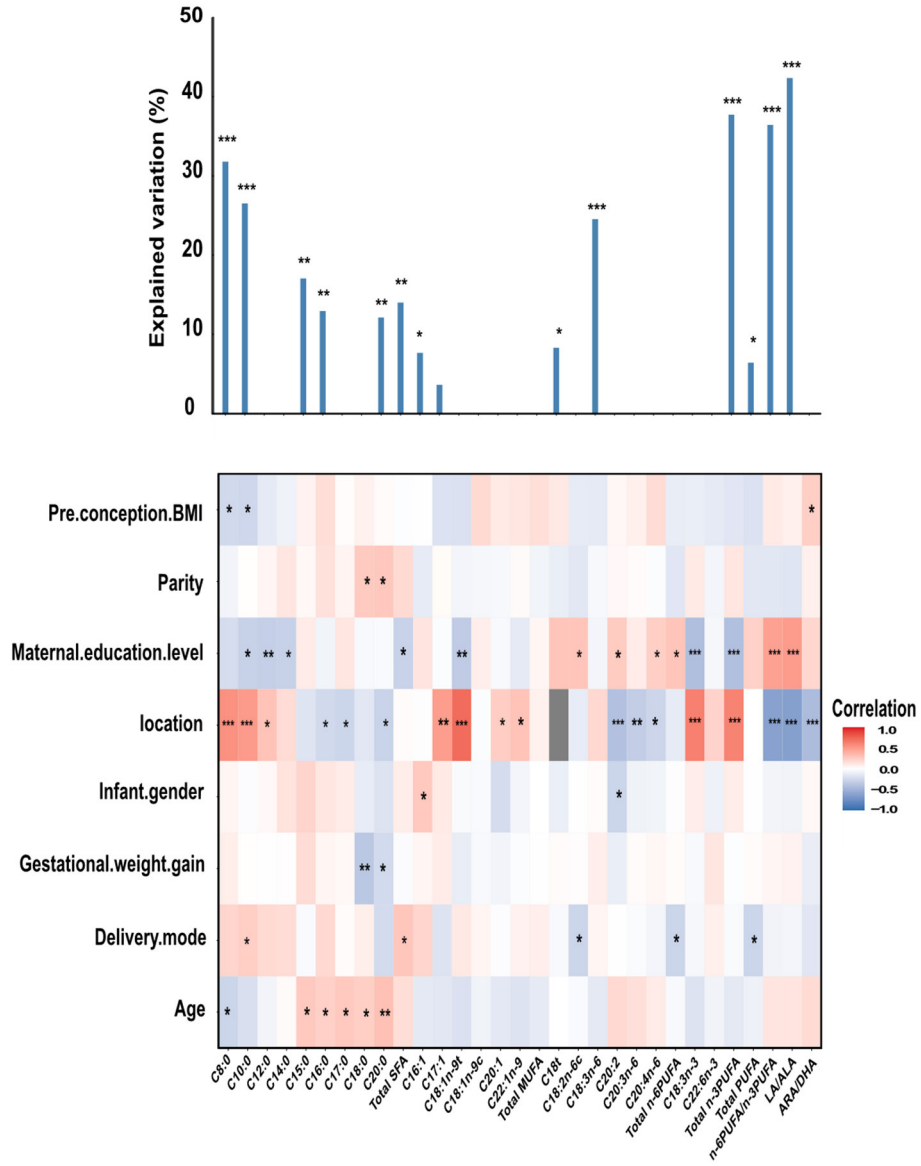

FIGURE 1
The column chart shows the total contribution of indicators to the interpretation of FAs variation (obtained by multiple linear regressions). The heat map shows the Spearman correlations between total FAs and correlated factors. Coloring reflects the direction and magnitude of the correlation coefficients. For pre-conception BMI, parity, maternal education level, gestational weight gain, and age, the higher the value/level of the correlated factors, the higher the fatty acid content. For the location, red indicates FAs in Danyang are higher than that in Beijing. For infant gender, red indicates FAs in mothers with baby girls are higher than that with baby boys. For delivery mode, red indicates FAs of mothers with natural childbirth are lower than with other modes of delivery. The major predictors were identified based on the correlation and best multiple regression model. C18t is the sum of C18:1t, C18:2t, and C18:3t, representing the level of trans FAs. LA, linoleic acid (C18:2n-6c); ARA, arachidonic acid (C20:4n-6); ALA, α-linolenic acid (C18:3n-3); DHA, Docosahexaenoic acid (C22:6n-3). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Discussion and conclusions

In this present study, we analyzed the total and sn-2 FA profiles in mature milk samples from healthy lactating women in Beijing and Danyang, China. We found specific associations of pre-conception BMI, maternal education level, location, infant gender, gestational weight gain, delivery mode, and maternal age with total and sn-2 FAs in mature milk. Parity may affect total FA composition but not sn-2 FA. Location and maternal education level were strong predictors for differences in the constitution of mature milk total and sn-2 FAs.

TABLE 3 Composition of sn-2 fatty acids and relative molar percentage[a] of each fatty acid in mature milk at the sn-2 position (%).

Lipids	sn-2 fatty acids		Relative molar percentage	
	Mean±SD	Range	Mean±SD	Range
C8:0	0.08 ± 0.03	0.02∼0.15	23.86 ± 11.13	11.10∼61.67
C10:0	0.80 ± 0.28	0.22∼1.75	27.66 ± 9.85	6.90∼64.87
C12:0	5.23 ± 1.96	1.18∼12.38	47.23 ± 9.49	10.89∼68.26
C14:0	6.83 ± 2.46	2.95∼15.05	66.80 ± 10.54	18.98∼90.55
C15:0	0.31 ± 0.09	0.18∼0.66	75.31 ± 11.04	45.11∼95.24
C16:0	50.18 ± 4.74	36.03∼61.42	79.72 ± 4.29	64.29∼86.82
C17:0	0.26 ± 0.06	0.15∼0.54	39.12 ± 6.51	23.92∼58.94
C18:0	3.79 ± 1.25	1.78∼7.60	22.35 ± 7.96	10.15∼47.51
C20:0	0.22 ± 0.09	0.11∼0.54	39.05 ± 13.79	15.10∼77.78
C22:0	0.05 ± 0.04	0.00∼0.17	24.55 ± 17.67	0.31∼80.45
C14:1	0.11 ± 0.17	0.02∼0.75	29.36 ± 12.82	10.26∼72.67
C16:1	2.72 ± 0.61	1.18∼4.48	46.56 ± 9.39	26.15∼68.30
C17:1	0.18 ± 0.05	0.06∼0.33	29.91 ± 9.56	11.02∼57.09
C18:1t	0.12 ± 0.10	0.04∼0.75	32.54 ± 23.49	9.81∼87.57
C18:1	12.78 ± 2.26	7.93∼22.43	11.86 ± 1.69	8.50∼17.14
C20:1n-9	0.30 ± 0.14	0.11∼0.92	16.34 ± 3.60	8.87∼30.25
C22:1n-9	0.17 ± 0.21	0.02∼0.91	25.84 ± 8.54	12.56∼49.40
C18:2t	0.18 ± 0.11	0.06∼0.84	21.21 ± 5.41	12.85∼42.30
C18:2n-6	13.49 ± 3.61	7.64∼29.91	17.85 ± 2.55	12.17∼24.57
C18:3n-6	0.12 ± 0.06	0.03∼0.45	27.48 ± 12.11	11.13∼83.74
C18:3t	0.16 ± 0.11	0.05∼0.78	34.89 ± 18.23	15.10∼81.34
C20:2n-6	0.18 ± 0.05	0.05∼0.31	12.48 ± 2.83	3.39∼18.04
C20:3n-6	0.14 ± 0.18	0.04∼1.09	8.02 ± 4.39	3.85∼25.73
C20:4n-6	0.30 ± 0.09	0.11∼0.56	17.23 ± 4.11	7.88∼25.74
C18:3n-3	1.20 ± 0.53	0.26∼2.82	23.31 ± 5.43	12.83∼49.81
C22:6n-3	0.33 ± 0.12	0.04∼0.62	37.79 ± 15.57	6.68∼80.62

[a]calculated as (M/T/3) ×100, M, molar percentage of sn-2 fatty acid; T, molar percentage of total fatty acid.

Fat content and total FAs

Total lipid content in mature breast milk found in our study was 3.47 ± 1.52 g/100 g, which was close to the level (3.39 ± 1.24 g/dl) found in a recent systematic review in Chinese women (31). Our study showed that the total contents of SFA, MUFA, and PUFA in mature milk were 34.49 ± 3.88%, 37.57 ± 3.82%, and 27.94 ± 4.36%, respectively, which was similar to the pooled results of the Asian populations in a meta-analysis study (37.87∼39.91%, 34.64∼36.72%, 23.18∼29.79% for SFA, MUFA, PUFA, respectively) (32). It is worth noting that the infant milk formula standards of China (GB10765-2010) specify that the ratio of LA/ALA should range from 5:1 to 15:1, which is in line with the standards of international organizations and other countries, e.g., the Food and Agriculture Organization of the United Nations, WHO, Australia and New Zealand (33). However, the mean value for LA:ALA in our study was 17.89:1; other studies conducted among the Chinese population also showed a mean value for LA/ALA in mature milk above 10:1 [Shanghai:18.56:1 (34); Guangzhou:19.70:1 (35); Beijing:15.69:1 (35), Jiangsu:11.9:1 (35)]. It is suggested that we should focus on the characteristics of domestic breast milk to guide the formulation and production of infant formula, rather than just referring to standards that are based on data from the breast milk of mothers in western countries. In our study, the sampling site had a significant effect on PUFAs. The levels of n-6 series of long-chain polyunsaturated fatty acids (LCPUFA) (C20:2, C20:3n-6, C20:4n-6, n-6PUFA/n-3PUFA, LA/ALA, and ARA/DHA) in mature milk from mothers in Beijing were higher than that of Danyang, while the opposite was observed in the n-3 series of LCPUFA (C18:3n-3 and Total n-3PUFA). The discrepancy in eating habits between the two regions might lead to the difference in the proportions of PUFA in the mature milk. Beijing is an inland city, while Danyang is a coastal city abundant in various fishes rich in n-3 PUFA. In addition to PUFA, regional differences were also observed in several types

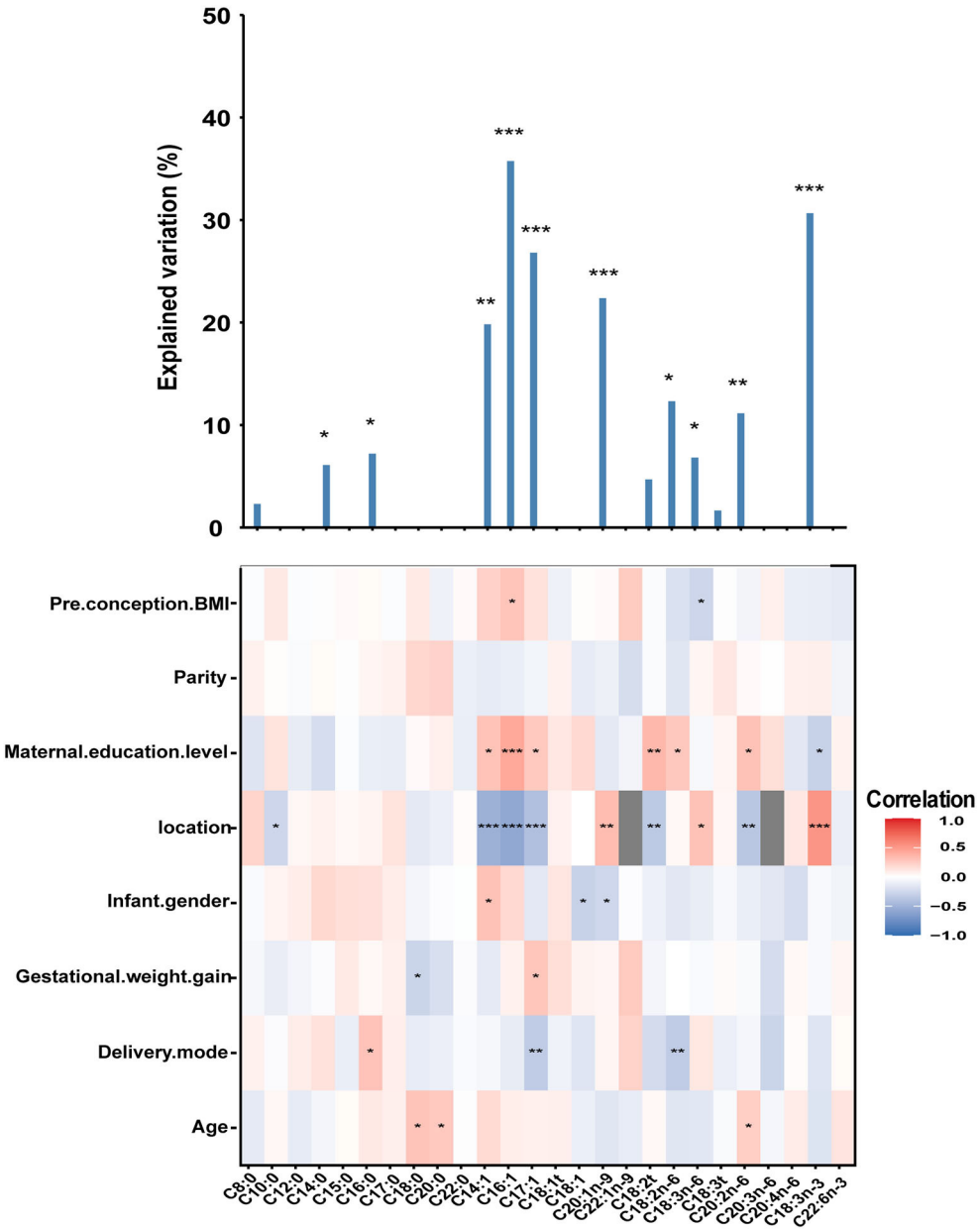

FIGURE 2
The column chart shows the total contribution of indicators to the interpretation of sn-2 FAs variation (obtained by multiple linear regressions). The heat map shows the Spearman correlations between sn-2 FAs and correlated factors. Coloring reflects the direction and magnitude of the correlation coefficients. For pre-conception BMI, parity, maternal education level, gestational weight gain, and age, the higher the value/level of the correlated factors, the higher the fatty acid content. For the location, red indicates FAs in Danyang are higher than that in Beijing. For infant gender, red indicates FAs in mothers with baby girls are higher than that with baby boys. For delivery mode, red indicates FAs of mothers with natural childbirth are lower than with other modes of delivery. The major predictors were identified based on correlation and the best multiple regression model. DHA, Docosahexaenoic acid (C22:6n-3). *$P < 0.05$; **$P < 0.01$; ***, $P < 0.001$.

of SFA (C8:0, C10:0, C12:0, C16:0, C17:0, and C20:0) and MUFA (C17:1, C18:1n-9t, C20:1, and C22:1n-9), which might be caused by other site-specific correlated factors, such as gene, climate, or lifestyle.

Maternal education level was also found to be associated with some human milk FAs. Mothers with higher degrees had lower SFAs (C10:0, C12:0, C14:0, and Total SFA), n-3 series of LCPUFA (C18:3n-3 and Total n-3PUFA), C18:1n-9t,

and higher n-6 series of LCPUFA (C18:2n-6c, C20:2, C20:4n-6, Total n-6PUFA, and n-6PUFA/n-3PUFA, LA/ALA ratios). In a similar manner, a study on low-income Indian women showed that a higher maternal education resulted in lower concentrations of SFAs and PUFAs (36). However, Al-Tamer and Mahmood (37) found the proportions of the n-3 series of LCPUFA decreased with decreasing socioeconomic status (mother's education and occupation). The effect of maternal education on the FA in human milk is equivocal. A lower level of education usually implies higher unemployment or lower wages and a lower income level (38). Besides, as a reflection of traditional gender roles in society, women are more likely to take on the responsibility of food selection and acquisition. Taking all into consideration, maternal education level may influence the nutritional knowledge and income levels, which may lead to the difference in food consumption and dietary habits, and therefore, affect the profiles of FAs.

The influence of maternal age on FAs is ambiguous. In this study, maternal age is positively associated with some SFAs (C15:0, C16:0, C17:0, C18:0, and C20:0). Antonakou et al. (39) also reported that maternal age was an independent factor of MUFAs. However, two studies (40, 41) reported that maternal age was not related to milk lipids. Moreover, the association between infant gender and FAs was observed in our study. Infant gender was reported to influence hormonal secretions in the placenta during pregnancy, which is related to breast development (42). This may help explain the associations found in our study.

The results also showed that the mature milk of mothers who had delivered with a natural birth contained fewer SFAs (C10:0 and Total SFA) and more PUFAs (C18:2n-6c, Total n-6PUFA, and Total PUFA) compared with the cesarean section group. Sinanoglou et al. investigated the factors affecting human colostrum FAs and found that the proportions of C12:0, C14:0, C18:3n-3, C20:4n-6, C20:5n-3, and Total n-3PUFA were significantly ($P < 0.05$) lower in colostrum fat from cesarean than from vaginal deliveries (43). Gestational weight gain, infant gender, parity, and pre-conception BMI were also found to be associated with several human milk FAs, suggesting a possible role between these characteristics in FAs. As few studies have examined the associations between maternal characteristics and FAs, more research is needed to clarify these factors.

Sn-2 FAs

The FAs at the sn-2 position of TAG in human milk were reported to significantly affect the absorption of FAs and calcium, infant intestinal flora (17), and stool consistency (44). TAGs with sn-2 FAs have recently become a target in the optimizing of infant formula (45–47).

Results of our study corresponded with Deng et al. (22), in which several SFAs (C14:0, C15:0, and C16:0) were mainly acylated in the sn-2 position, and most MUFAs and PUFAs showed sn-1,3 positional selectivity in TAGs.

Notably, the sn-2 FA profile seemed to be less affected by the factors that influence total FA. Parity was not found to be associated with sn-2 FAs. The association between location and several FAs (C20:1n-9, C18:3n-3, and C20:2n-6) in total FAs was similar to that in sn-2 FAs. The sn-2 FAs as a part of total FAs may account for the similarity regarding these correlations between total and sn-2 FAs. C16:0 and DHA are proved to be beneficial to infants' health under the sn-2 positional selectivity (17, 44, 48). However, DHA was not observed to be associated with the factors discussed earlier, and C16:0 was only found to be associated with the delivery mode. This may suggest that the levels of the two FAs are relatively constant. The average levels of C16:0 and DHA at the sn-2 position among the domestic population may be a reliable reference for their infant milk powder formulation.

Based investigation of the profiles of FAs and sn-2 FAs in human mature milk samples from two representative areas in China, this study also strived to explore the associations between maternal factors and FAs, especially, sn-2 FAs, which are rarely explored in previous studies. Our study has a few limitations. First, a systematic dietary survey was not conducted, so nutrient intakes cannot be estimated, and the effects of maternal diet on FAs cannot be explored. In addition, we restricted our analyses to several sociodemographic factors, but other elements, namely, lactation stage, genes, and gestational age, were also reported to influence the FA profile. Further research is needed to integrate all the correlated factors. Despite these limitations, one of the outstanding advantages of this study is that the sampling sites were restricted to the hospital, and breast pump sampling was used to reduce the confounding factors of sampling, which significantly improved the reliability of the results.

In conclusion, this study elucidated the total and sn-2 FA profiles of mature milk in women from Beijing and Danyang, China. Correlation analysis revealed that the total FAs composition was variable and independently associated with location, maternal age, infant gender, pre-conception BMI, gestational weight gain, delivery mode, parity, and maternal education level. On the contrary, sn-2 FAs composition seemed more constant than total FAs, as parity was not found to be associated with the levels of sn-2 FAs. The conspicuous contribution of location and maternal education level was observed in both total and sn-2 FAs, which implicated the possible role of economic–related or education–related dietary habits; the delivery mode was

also a significantly correlated predictor of the variation in FAs and sn-2 FAs. Together, these findings present a pilot study on the correlated factors of FAs in mature milk and may act as a reference for infant formula or human milk fortifier optimization.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Chinese Clinical Trial. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MN conducted the experiments, analyzed the data, and wrote the first draft of the manuscript. YW contributed to the conception and design of the study. ZY wrote a part of the first draft. XX and HZ provided experimental and technical support. JC contributed to manuscript revision, read, and approved the submitted version. YY and LZ supervised. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors XX and HZ were the employees of the Wilmar (Shanghai) Biotechnology Research & Development Center Co., Ltd. when this work was done. They participated in the validation of the study and the opinions they expressed were their own and do not necessarily reflect the views or recommendations of their respective affiliations.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.926429/full#supplementary-material

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Glucocorticoids in preterm human milk

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Background: Glucocorticoids (GCs), cortisol and cortisone, are essential regulators of many physiological responses, including immunity, stress and mammary gland function. GCs are present in human milk (HM), but whether maternal and infant factors are associated with HM GC concentration following preterm birth is unclear.

Materials and methods: HM samples were collected on postnatal day 5 and 10 and at 4 months' corrected age (4m CA) in a cohort of moderate- and late-preterm infants. GCs in HM were measured by liquid chromatography-tandem mass spectrometry. Relationships between GCs in HM and both maternal and infant characteristics were investigated using Spearman's correlations and linear mixed models.

Results: 170 mothers of 191 infants provided 354 HM samples. Cortisol concentrations in HM increased from postnatal day 5–4m CA (mean difference [MD] 0.6 ± 0.1 ng/ml, $p < 0.001$). Cortisone concentration did not change across lactation but was higher than cortisol throughout. Compared to no antenatal corticosteroid (ANS), a complete course of ANS was associated with lower GC concentrations in HM through to 4m CA (cortisol: MD −0.3 ± 0.1 ng/ml, $p < 0.01$; cortisone MD −1.8 ± 0.4 ng/ml, $p < 0.001$). At 4m CA, higher maternal perceived stress was negatively associated with GC concentrations in HM (cortisol adjusted beta-coefficient [aβ] −0.01 ± 0.01 ng/ml, $p = 0.05$; and cortisone aβ −0.1 ± 0.03 ng/ml, $p = 0.01$), whereas higher postpartum depression and maternal obesity were associated with lower cortisone concentrations (aβ −0.1 ± 0.04 ng/ml $p < 0.05$; MD [healthy *versus* obese] −0.1 ± 0.04 ng/ml $p < 0.05$, respectively). There was a weak positive correlation between GC concentrations in HM and gestational age at birth ($r = 0.1$, $p < 0.05$). Infant birth head circumference *z*-score was negatively associated with cortisol concentrations (aβ −0.01 ± 0.04 ng/ml, $p < 0.05$). At hospital discharge, fat-free mass showed a weak positive correlation with cortisol concentrations ($r = 0.2$, $p = 0.03$), while fat mass showed a weak negative correlation with cortisone concentrations ($r = -0.25$, $p < 0.001$).

Conclusion: The mammary gland appears to protect the infant from cortisol through inactivation into cortisone. Maternal and infant characteristics were associated with concentration of GCs in HM, including ANS, stress and depression scores, obesity, gestational age and infant size. The effects of HM glucocorticoids on long-term health outcomes requires further research.

KEYWORDS

cortisol, cortisone, breastmilk, lactation, nutrition, antenatal corticosteroids, moderate preterm, late preterm

Introduction

Human milk (HM) can act as a messenger between mother and the infant, conveying nutritional, non-nutritional, immune, and biologically active compounds involved in various signaling pathways key for development (1–3). Hormones in HM confer individualized cues about maternal health and environment that may influence infant metabolism, growth and postnatal development with potential long-term effects (3–5).

The hypothalamic-pituitary-adrenal (HPA) axis is essential for orchestrating response to stressors and the circadian cycle (6). During pregnancy, the maternal HPA axis undergoes dramatic changes, with cortisol concentrations progressively rising to a peak in the third trimester, partially in response to increasing secretion of placental corticotrophin-releasing hormone (CRH) (6, 7). To protect the fetus from excessive exposure to cortisol, the placenta expresses 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an enzyme that converts cortisol into its inactive form, cortisone (7, 8). By late gestation (around 34 weeks), fetal adrenal production of the glucocorticoids (GCs) cortisol and cortisone gradually increases and is essential for maturation of fetal organs including lungs, thyroid and the gastrointestinal tract, in preparation for the postnatal environment (8, 9). For mothers at risk of preterm birth, administration of antenatal glucocorticoids accelerates fetal lung maturation and reduces neonatal morbidity and mortality (10). Following birth, circulating maternal cortisol concentrations and HPA axis activity gradually return to pre-pregnancy levels (7, 11).

Data from animal and human research suggest that exposure to endogenous (i.e., hypertension, insulin resistance, undernutrition, obesity) and exogenous (i.e., depression, social deprivation) maternal stressors during pregnancy and the perinatal period is associated with a higher risk for obesity and metabolic dysfunction in the offspring (6, 9). Maternal stress/anxiety, and inflammation or infection during pregnancy can result in excessive maternal cortisol concentrations and, although 11β-HSD2 is capable of metabolizing up to 90% of maternal cortisol, there may be increased fetal exposure to GCs during the critical period of HPA axis development (7, 8). Considering the central role of the HPA axis in key metabolic pathways, insults during HPA axis development are proposed as a mechanism by which adverse perinatal exposures may influence life-long health (6, 9).

Together with prolactin and insulin, maternal GCs also play a central role in lactogenesis, stimulating mammary gland differentiation and structural changes to support HM synthesis and secretion (12). The GCs cortisol and cortisone are two of many hormones present in HM (3–5). Concentrations of cortisol and cortisone in HM follow the maternal HPA axis activity (13–15), displaying the same circadian rhythm, peaking in the morning and slowly decreasing throughout the day (13, 14, 16). A diurnal rhythm of GCs is present in term and preterm infants from 1 month postnatal and corrected age, respectively (17, 18). The establishment of circadian rhythm is essential to control normal sleep–wake cycles, respiratory rate, body temperature, digestion, metabolism, hormone release, and other important physiological functions (5, 6). However, compared to full-term infants, those infant born extremely preterm (<28 weeks) have blunted cortisol reactivity to acute stress at 4 months chronological age (19) and flattened diurnal cortisol slope across the first year of life (20), possibly indicating suppression of HPA axis activity.

Whether GCs in HM relate to maternal and infant health remains unclear, with studies reporting conflicting associations between GCs in HM and the effects of lactation stage (21–23), gestational age (13, 21, 24, 25), maternal stress (16, 24, 26), infant adiposity (27, 28) and temperament (23, 29). Moreover, only small studies have investigated the concentration of GCs in preterm HM and mainly after very preterm birth (<32 weeks' gestation) (13, 21, 25). Therefore, the aim of the present study was to determine HM concentrations of cortisol and cortisone from mothers of moderate to late preterm infants and explore potential associations with maternal and infant characteristics.

Materials and methods

Study population

We undertook a prospective cohort analysis nested within the DIAMOND trial (ACTRN12616001199404; Health and Disability Ethics Committee 16/NTA/90) (30). Briefly, this

factorial randomized controlled trial compared three nutritional interventions in moderate and late preterm infants admitted to four neonatal nurseries in New Zealand. The interventions were: (i) dextrose solution *versus* amino acid solution (with or without lipid emulsion at the clinician's discretion) as the intravenous fluid; (ii) breastmilk as the only milk *versus* a breastmilk substitute if maternal breastmilk supply did not meet infant requirements, and (iii) exposure of the infant to smell and taste of milk prior to each feed by gastric tube, *versus* no exposure. Eligibility criteria were: birth between 32^{+0} and 35^{+6} weeks' gestation; intravenous lines *in situ*; mother intending to breastfeed, and no congenital abnormality that might impact upon growth and development.

Maternal age, ethnicity, education, postcode, and clinical information (maternal diabetes, antenatal steroid administration, delivery mode), and infant characteristics (sex, gestational age at birth, anthropometric measures) were collected prospectively. In New Zealand, postcode of domicile is used to generate a social deprivation index (the New Zealand Deprivation [NZdep] Index) in the Classification Coding System from Statistics New Zealand (31) with a decile scale from 1 to 10, representing low to high social deprivation. Ethnicity was self-identified and prioritized according to New Zealand's Ministry of Health protocols (32). For analysis, ethnicity was grouped into Caucasian/European; Asian (Asia, South-East Asia, and Indian subcontinent); Pasifika (South-Western Pacific); Māori (New Zealand Māori); and Other. Antenatal corticosteroid (ANS) administration was classified as a complete course (>1 dose given, with the first dose >24 h before birth), incomplete (1 dose given <24 h before birth), or no ANS received.

Two self-completed questionnaires were used to assess symptoms of depression (Edinburgh Postnatal Depression Scale, EPDS) and stress (Perceived Stress Scale, PSS) around postnatal day 10 and when the infant was 4 months' corrected age (4m CA, ± 2 weeks, defined from 40 weeks' gestation). The EPDS asked how mothers felt during the previous week prior to completion of the questionnaire. The maximum score is 30 and mothers who scored 10 or above possibly experienced symptoms of postnatal depression (33, 34). The PSS questionnaire asked how mothers felt during the previous month using a 14-item instrument. Scores ranging from 0 to 13, 14 to 26, and 25 to 40 points were considered low, moderate and high perceived stress, respectively (35).

Nutrition and growth

Data about nutritional intake and growth during hospital stay were collected prospectively. Weight, length and head circumference were recorded weekly until hospital discharge and again at 4m CA. *Z*-scores were calculated based on the Fenton preterm growth charts for in-hospital growth (36) and on the World Health Organization (37) growth chart at 4m CA. Linear growth was calculated based on changes in *z*-score between two time points of interest. Growth velocity from birth to discharge was calculated using the exponential method (38). Body composition was measured by air displacement plethysmography (PEA POD®, COSMED, Concord, CA, USA) in a sub-set of infants at hospital discharge and at 4m CA.

Sample collection

HM samples were collected during the morning on postnatal day 5 (±2 days) and 10 (±2 days) and at 4m CA (±2 weeks) if mother was still breastfeeding. Mothers were requested to express milk from their right breast using an electronic breast-pump (Medela Symphony®, Switzerland) into disposable sterile bottles (Medela®, Switzerland) at least 2–3 h after the previous milk expression. After the right breast was completely emptied, the total volume of expressed HM was vortexed for 2 min at high speed to ensure homogeneity and 2 ml was collected using a sterile enteral syringe (BD, Singapore). The collected HM was aliquoted into low-protein-binding microtubes (Eppendorf, Germany) and stored at –80°C until analysis. The lactation stage was defined as colostrum (samples collected around postnatal day 5), transitional HM (samples collected around postnatal day 10), and mature HM (samples collected at 4m CA). The cohort reported here includes mothers who provided at least one HM sample during the study period and their infants.

Analysis of glucocorticoids in preterm human milk

HM GCs were measured by liquid chromatography-tandem mass spectrometry, as described previously (15). Briefly, 200 μl of HM heated at 37°C for 10 min was vortexed for 20 s before adding sample to glass tubes with internal standards (12 ng/ml cortisol D4 and 60 ng/ml corticosterone D8, prepared in milli-Q water). Steroids were then extracted using 1 ml ethyl acetate (Merck, Germany); the top organic layer was removed and vacuum dried (Savant, SC250EX, Thermo Scientific, USA) for 1 h, and then reconstituted with 60 μl 50% methanol (Merck, Germany) in water and transferred to Ultra High Pressure Liquid Chromatography (UPLC) injector vials. The UPLC Mass Spectrometer (MS) used a Vanquish pump and auto-sampler followed by an Ion Max APCI source on a Quantiva triple-quadrupole mass spectrometer, controlled by Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA). The chromatography was performed using Phenomenex C18 F5 column (100 × 2.1 mm, 2.6 μm particle size) at 40°C.

GC concentrations were calculated from a standard curve generated for each GC relative to its internal standard, cortisol 0.05–100 ng/ml and cortisone 0.025–50 ng/ml, diluted into

charcoal stripped human plasma (SeraCon II CD, Seracare, Milford MA, USA) and extracted in the same way as the samples for each assay. The limits of quantification for cortisol and cortisone were 0.05 and 0.025 ng/ml, respectively. The limits of detection for cortisol and cortisone were 0.03 and 0.01 ng/ml, respectively.

Statistical analyses

Concentration of cortisol and cortisone are presented in ng/ml of HM. Concentrations of GCs were assessed for normality using Shapiro–Wilk test. Logarithmic transformation did not result in a normal distribution, and therefore descriptive analyses were performed with and without logarithmic transformation. As similar results were obtained, further statistical analyses were performed without logarithmic transformation. Spearman correlation was used to assess the relationship between GC concentrations and maternal and infant characteristics, and these were then further explored using linear mixed models including maternal study number as a random effect and lactation stage as a fixed effect and adjusted for ANS course. Models exploring infant factors were further adjusted for infant sex. Longitudinal changes in GC concentrations were explored using linear mixed models including maternal study number (within-subjects effect) and ANS course (fixed effect). Missing datapoints were estimated using restricted maximum likelihood. Statistical analyses were performed in R v4.1.0 software (R Core Team, Vienna, Austria) using the base "*Stats*" library (v3.6.1). Linear mixed models were conducted using R library "*lme4*" (v 1.1-26) and *post hoc* tests were conducted using R library "*emmeans*" (v1.5.5). A p value of ≤ 0.05 was considered significant, with false discovery rate-adjusted p value (FDR, Tukey Honest Significant Difference) for multiple comparisons. Data are presented as frequency (%), mean (standard deviation, SD) or median (range, minimum–maximum).

Results

Study population

In total, 230 babies were enrolled in the DIAMOND trial between March 2017 and August 2019. Of these, 171 mothers of 192 infants provided samples HM samples. One mother-infant pair was excluded from analysis due to extremely high HM cortisol and cortisone concentrations (23 and 20.5 ng/ml, respectively), leaving 170 mothers of 191 babies who provided 354 HM samples (day 5: $n = 149$, day 10: $n = 140$ and 4m CA: $n = 65$) included in this analysis (**Figure 1**). Mean (standard deviation, SD) postnatal age at HM sample collection was 5 (1), 9 (1) and 160 days, and 70% of samples were collected before midday. Most mothers received at least some ANS (79%), had at least post-secondary education (70%) and were of Caucasian or Asian ethnicity, with Māori ethnicity under-represented compared with national birthing data (12 vs 25% nationally; **Table 1**). Mothers residing in areas of high social deprivation (NZDep quintiles 4 and 5) were over-represented (47%) and 60% were overweight or obese. Infants were born at a median (range) gestational age of 33 weeks, 12% were small-for-gestational age (SGA), 26% were multiples and 57% were boys (**Table 1**). Average (SD) length of hospital stay was 22 days and 78% of mothers were providing exclusively HM to their babies at hospital discharge (breastfeeding and/or expressed HM). However, only 19% of mothers were exclusively breastfeeding at 4m CA (**Table 2**).

Lactation stage

The median concentration of cortisol in preterm HM was 0.5 (range 0.03–3.7) ng/ml and of cortisone was 4.3 (0.3–15.5) ng/ml, with cortisone concentrations higher than cortisol concentrations throughout. Cortisol concentration was significantly higher in mature HM than in samples collected on days 5 and 10 (mean (standard error [SE]) *day 5*: 0.6 (0.05) ng/ml; *day 10*: 0.5 (0.05) ng/ml; *4m CA*: 1.2 (0.1) ng/ml, $F_{(2,\ 248)} = 35.7$, $p < 0.001$; **Figure 2A**). In contrast, cortisone concentrations did not change significantly over time (*day 5*: 5.2 (0.2) ng/ml; *day 10*: 5.0 (0.2) ng/ml; *4m CA*: 4.5 (0.3) ng/ml, $F_{(2,\ 234)} = 2.2$, $p = 0.1$). The ratio between cortisol and cortisone increased from early to late lactation ($p < 0.001$; **Figure 2A**). Concentrations of cortisol and cortisone were highly correlated throughout lactation; however, the strength of the correlation decreased as lactation progressed (**Figure 2B**).

Total volume of HM expressed at time of sample collection on days 5 and 10, but not 4m CA, showed a weak negative correlation with cortisol concentrations (*day 5*: $r = -0.2$, $p = 0.01$; *day 10*: $r = -0.2$, $p = 0.03$; *4m CA*: $r = -0.03$, $p = 0.7$) and cortisol-to-cortisone ratio (*day 5*: $r = -0.2$, $p < 0.001$; *day 10*: $r = -0.2$, $p < 0.01$; *4m CA*: $r = 0.1$, $p = 0.4$). Cortisone concentration was not correlated with total volume of HM expressed (*day 5*: $r = -0.1$, $p = 0.4$; *day 10*: $r = 0.04$, $p = 0.6$; *4m CA*: $r = -0.1$, $p = 0.6$).

Perinatal factors

Mothers who received a complete course of ANS had significantly lower concentrations of cortisol (mean difference [MD] –0.3 (0.1) ng/ml, $p < 0.01$) and cortisone (MD –1.8 (0.4) ng/ml, $p < 0.001$) in HM than mothers with no ANS, regardless of length of gestation (moderate *versus* late preterm birth), and this association persisted to 4m CA (**Figure 3A**).

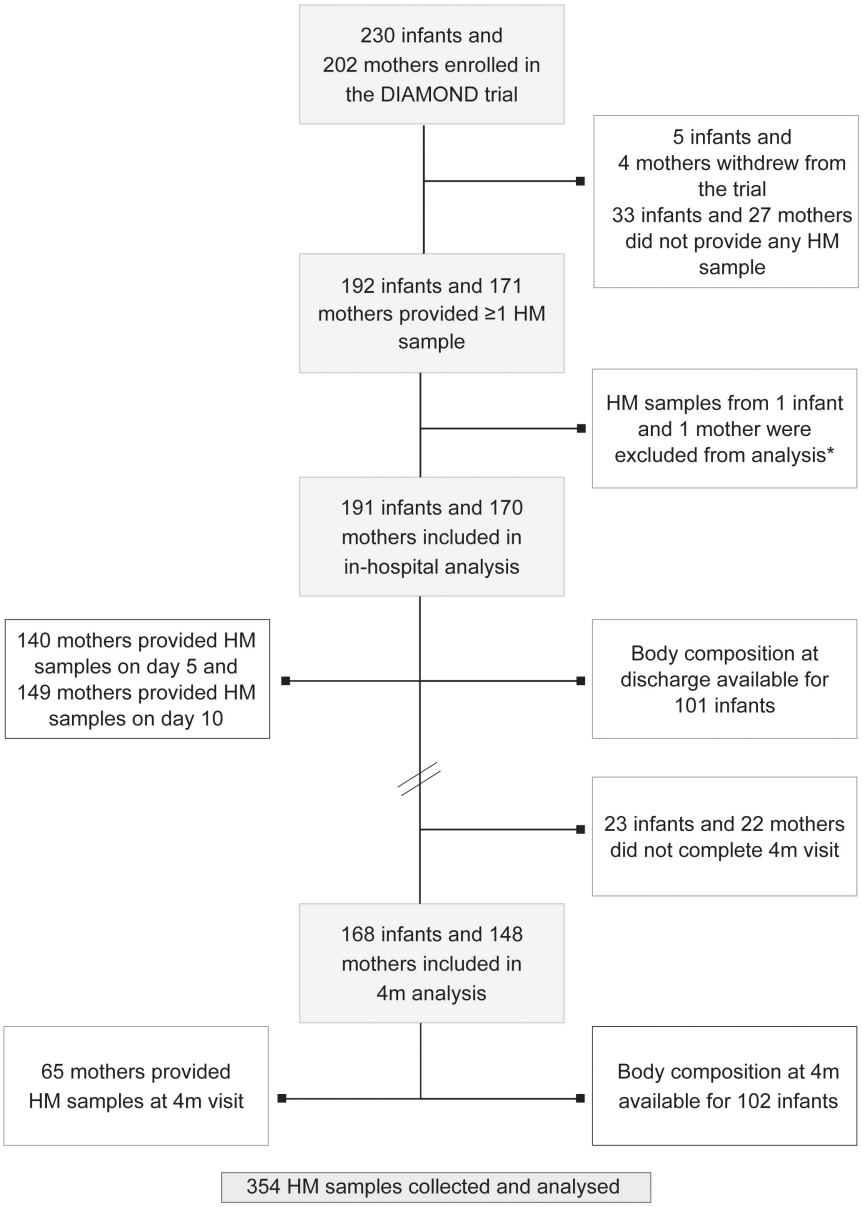

FIGURE 1
Study flowchart. Note that numbers do not add up as some mothers provided >1 sample and some mothers gave birth to multiples (twins and triplets). HM: human milk; 4m: 4 months follow-up visit. *Outlier sample excluded from analysis.

Overall, there was no significant association between GC concentration in HM and mode of birth (p = 0.7; Supplementary Table 1); however, mothers who birthed vaginally and received ANS had lower HM cortisol concentrations than mothers who birthed vaginally and received no ANS through to 4m CA (MD −0.5 (0.1) ng/ml, p = 0.04; mode of birth * ANS interaction $F_{(2,155)}$ = 3.9, p = 0.02; Figure 3B). Although the pattern was similar for cortisone between mothers who birthed vaginally and received ANS and those who birthed vaginally and received no ANS, this was not statistically significant (MD −2.1 (0.6) ng/ml, p = 0.09; birth mode * ANS interaction $F_{(2,160)}$ = 2.3, p = 0.1). Among women who birthed by Caesarean section, there were no differences in cortisol or cortisone concentrations between those who had and had not received ANS (Figure 3B).

Compared to mothers of singletons, HM from mothers of twins or triplets had higher cortisone concentrations (MD = 1.1 (0.4) ng/ml; $F_{(1,164)}$ = 7.0, p = 0.01, Figure 3C). The concentration of GCs in preterm HM were not associated with maternal age, ethnicity, socioeconomic deprivation, level of education or maternal diabetes (Supplementary Table 1).

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TABLE 1 Study population characteristics.

Maternal characteristics (n = 170)	
Age, years (mean, SD)	31 (6)
Ethnicity	
Caucasian	66 (39)
Māori	20 (12)
Asian	54 (32)
Pasifika	27 (15)
Other	3 (2)
New Zealand Deprivation Index	
Q1 (1,2)	27 (16)
Q2 (3,4)	39 (23)
Q3 (5,6)	24 (14)
Q4 (7,8)	30 (18)
Q5 (9,10)	40 (29)
Education level	
Secondary education or lower	51 (30)
Post-secondary education	30 (18)
Tertiary education (university degree or higher)	89 (52)
Number of samples at each time point	
Day 5	149 (42)
Day 10	140 (39)
4 months	65 (18)
Pregnancy characteristics (n = 170)	
Antenatal steroid course	
None	35 (21)
Incomplete (first dose <24 h from birth)	38 (22)
Complete	97 (57)
Maternal diabetes	35 (21)
Caesarean section	101 (59)
Maternal BMI at 4 months (n = 134)	
Healthy (BMI ≤24.9 Kg/m^2)	53 (40)
Overweight (BMI 25-29.9 Kg/m^2)	32 (23)
Obese (BMI ≥30 Kg/m^2)	49 (37)
Infant characteristics (n = 191)	
Boys	109 (57%)
Gestational age, weeks (median, IQR)	33 (32–35)
Moderate preterm (32^{+0} to 33^{+6} weeks' gestation)	100 (52%)
Size at birth	
SGA	24 (12)
AGA	160 (84)
LGA	7 (4)
Twins/Triplets	50 (26)
Duration of hospital stay, days	22 (11)

Data are n (%) unless otherwise stated. Q, quintile of social deprivation; BMI, body mass index; IQR, interquartile range; SGA, small-for-gestational age; AGA, appropriate-for-gestational age; LGA, large-for-gestational age.

TABLE 2 Feeding practices and infant growth.

Feeding practices n (%)		
At discharge (n = 168)		
Exclusively HM	133 (78)	
Mainly HM (≥50% HM)	18 (11)	
Mainly IF (<50% HM)	12 (7)	
Exclusively IF	5 (3)	
At 4 months (n = 148)		
Exclusively HM	28 (19)	
Partially breastfeeding (HM + food, no IF)	20 (13)	
Mixed feeding (HM + IF + foods)	37 (25)	
IF feeding	63 (43)	
Anthropometric measures		
At birth (n = 191)		***z*-score**
Weight, g	2,102 (420)	−0.1 (0.9)
Length, cm	44.5 (3.0)	0.2 (1.1)
Head circumference, cm	31.2 (1.6)	0.3 (0.9)
At hospital discharge (n = 189)		
Weight, g	2,515 (330)	−0.8 (0.8)
Length, cm	47.5 (2)	−0.1 (0.9)
Head circumference, cm	33.0 (1.2)	−0.03 (0.7)
At 4m CA (n = 168)		
Weight, g	6,580 (867)	−0.1 (1)
Length, cm	63.7 (2.5)	0.5 (1.1)
Head circumference, cm	41.6 (1.3)	0.6 (0.9)
Growth birth to discharge, median (min, max)		
Growth velocity, g/Kg/day	7.7 (−19.3, 19)	
Weight z-score change	−0.7 (−1.8, 0.5)	
Length z-score change	−0.4 (−2.5, 2.3)	
Head circumference z-score change	−0.4 (−2.2, 1.4)	
Growth birth to 4m CA, median (min, max)		
Weight z-score change	−0.03 (−2.8, 2.1)	
Length z-score change	0.3 (−2.8, 4.8)	
Head circumference z-score change	0.3 (−2.1, 2.4)	
Body composition at discharge (n = 101)		**Kg**
Fat mass, %	10 (4)	0.25 (0.1)
Fat-free mass, %	90 (4)	2.2 (0.2)
Fat mass index, Kg/m^2	1.1 (0.6)	
Fat free mass index, Kg/m^2	9.8 (0.6)	
Body composition at 4m CA (n = 102)		**Kg**
Fat mass, %	25.3 (5)	1.6 (0.4)
Fat-free mass, %	74.7 (5)	4.8 (0.5)
Fat mass index, Kg/m^2	4.1 (1.0)	
Fat free mass index, Kg/m^2	11.8 (0.9)	

Data presented as n (%) or mean (SD), unless stated otherwise. HM, human milk; IF, infant formula; 4m CA, 4 months corrected age.

Maternal stress and postnatal depression

Median maternal PSS score was 15 (2–29) at day 10 and 13 (0–36) at 4m CA, with 5% of mothers having scores indicative of high level of stress at day 10 and 3% at 4m CA (Supplementary Table 1). GC concentration in HM was not different amongst mothers with different perceived levels of stress at either time point (Supplementary Table 1). PSS total scores at hospital discharge was not correlated with GC concentration in HM (Figure 4B). PSS total score at 4m CA exhibited a weak negative correlation with GC concentration in HM, which was statistically significant for cortisone (r = −0.1, p = 0.01) but not cortisol (r = −0.1, p = 0.2; Figure 4C). In the adjusted model, total PSS score at 4 months was negatively associated with HM concentrations of both cortisol (adjusted beta coefficient [aβ] −0.01 (0.01) ng/ml, $F_{(1,\ 118)}$ = 3.7, p = 0.05) and cortisone (aβ −0.1 (0.03) ng/ml, $F_{(1,\ 125)}$ = 6.2, p = 0.01).

Median scores on the EPDS were 7 (0–19) at day 10 and 5 (0–25) at 4m CA, with approximately 29% of mothers scoring above the threshold indicative of postnatal depression (scores ≥10) at day 10 and 22% at 4m CA (**Supplementary Table 1**). GC concentration in HM was not different between mothers who did and who did not have postnatal depression symptoms at day 10 (**Supplementary Table 1**) and there was no correlation between day 10 total EPDS score and GC concentration in HM (**Figure 4D**). There was a weak negative correlation between total EPDS score at 4m CA and GC concentration in HM, which was statistically significant for cortisone ($r = -0.1$, $p = 0.01$), but not cortisol ($r = -0.1$, $p = 0.2$; **Figure 4E**). In adjusted model, total EPDS score at 4m CA was negatively associated only with cortisone in preterm HM (aβ −0.1 (0.04) ng/ml, $F_{(1,\ 131)} = 5.7$, $p = 0.02$).

Maternal weight, height and body mass index

At 4m CA, maternal weight exhibited a weak negative correlation with GC concentration in HM, which was statistically significant for cortisone ($r = -0.1$, $p = 0.03$) but not cortisol ($r = -0.1$, $p = 0.09$). Maternal BMI also exhibited a weak negative correlation with GC concentrations in HM (cortisol: $r = -0.1$, $p = 0.03$; cortisone $r = -0.2$, $p < 0.01$). After model adjustment, maternal BMI was significantly associated only with cortisone concentrations ($F_{(2,\ 134)} = 3.1$, $p = 0.05$). Compared to mothers with healthy BMI, obese mothers produced HM with significantly lower cortisone concentrations (MD −1.0 (0.4) ng/ml, $p = 0.04$; **Figure 4A**).

Feeding practices

At hospital discharge, almost 80% mothers were feeding their infant with only HM (either expressed HM or breastfeeding; **Table 2**). There was no association between GC concentrations in preterm HM and feeding practices at discharge and at 4m CA (data not shown).

Infant factors

Both cortisol and cortisone concentrations in HM showed weak positive correlations with gestational age at birth (both $r = 0.1$, $p < 0.05$). Moderately preterm infants (32^{+0} to 33^{+6} weeks' gestation) tended to receive HM with lower cortisone concentration than late preterm infants (34^{+0} to 35^{+6} weeks' gestation; *cortisol* MD −0.1 (0.1) ng/ml, $p = 0.07$; *cortisone*: MD −0.4 (0.4) ng/ml, $p = 0.03$); however, these associations were no longer statistically significant after model adjustment (both adjusted $p = 0.2$; **Supplementary Table 2**). There were no differences in GC concentrations in preterm HM for girls and boys (**Figure 5A**).

Size at birth

GC concentrations in HM were not significantly correlated with weight and length at birth. In adjusted model, cortisol was negatively associated only with birth head circumference (HC) *z*-score (aβ −0.01 (0.04) ng/ml, $F_{(1,173)} = 4.2$, $p = 0.03$; **Supplementary Table 2**).

Growth

Changes in weight and HC *z*-scores between birth and discharge showed weak negative correlation with cortisone concentrations in HM ($r = -0.2$, $p < 0.001$; and $r = -0.1$, $p = 0.049$, respectively, data not shown). Growth between hospital discharge and 4m CA was not correlated with cortisol concentration in HM (all $r < 0.4$, $p > 0.1$, data not shown). There were no associations between growth and GC concentrations in preterm HM (**Supplementary Table 2**).

Infant body composition

Body composition was assessed in a subset of participants at hospital discharge ($n = 101$) and at 4m CA ($n = 102$). Mean (SD) fat and fat free mass percentage at discharge were 10 (4)% and 90 (4)%, respectively, and at 4m CA were 25 (5)% and 75 (5)%, respectively (**Table 2**). At hospital discharge, absolute fat free mass showed a weak positive correlation with cortisol concentrations in HM ($r = 0.2$, $p = 0.03$), while fat mass showed a weak negative correlation with cortisone concentrations ($r = -0.25$, $p < 0.001$; **Figure 5B**). Body composition at 4m CA was not correlated with GC concentration in HM (all $r < 0.2$, $p > 0.4$; **Figure 5C**). There were no associations between body composition at either age and GCs in preterm HM (**Supplementary Table 2**).

Discussion

In this longitudinal cohort of moderate to late preterm infants, we demonstrate that ANS administration is associated with lower concentrations of GCs in HM for at least 4 months, particularly following vaginal birth. ANS are widely used for prevention of neonatal morbidity among mothers at risk of preterm birth (10), but a significant proportion of women who receive antenatal glucocorticoids then go on to birth at, or near, term (39, 40). However, the potential effect of ANS on lactogenesis are seldomly investigated. We also confirm that

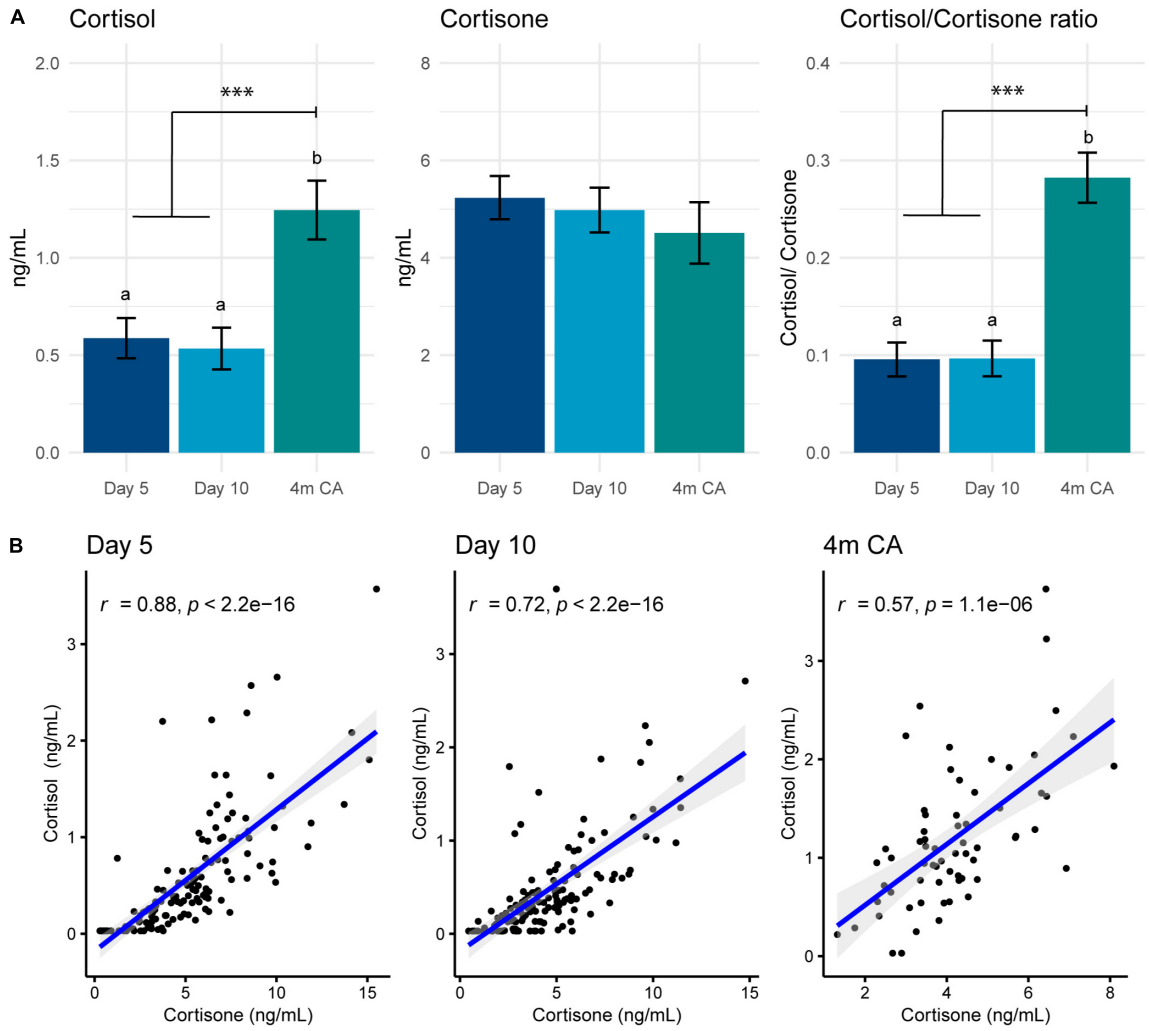

FIGURE 2
(A) Human milk concentrations of cortisol, cortisone and the cortisol-to-cortisone ratio at different lactation stages. Data are mean and standard error. Statistically significant differences are represented by different letters above the bars. ***$p < 0.001$ **(B)** Correlations between cortisol and cortisone at different lactation stages: postnatal day 5 (left), day 10 (middle), and 4 months' corrected age (right). Blue line represents Spearman correlation coefficient and shaded area the 95% confidence interval. 4m CA: 4 months' corrected age.

cortisone is the predominant GC in preterm HM through to 4m CA, indicating that the mammary gland may inactivate cortisol into cortisone. Our findings indicate that concentrations of GCs in preterm HM are associated with many maternal and infant characteristics, including maternal obesity, postnatal stress and depression scores, infant gestational age, head circumference at birth and body composition at hospital discharge

The development of the mammary gland is guided by "hormonal switches" during pregnancy and the postnatal period and both the order and timing of each hormonal exposure are essential for normal mammary development and lactation (12, 41). Mammary GC receptors increase during pregnancy and peak around the time of birth, remaining high during lactation until involution of the mammary gland at weaning (12), highlighting the essential role of GCs for mammary development (12, 42). During Lactogenesis I (or secretory differentiation), GCs act in synergy with prolactin and insulin in the mammary epithelial cells to induce differentiation of the lobule-alveolar system, development of rough endoplasmic reticulum and tight junctions, and expression of enzymes and milk protein genes, all of which are mechanisms essential for successful lactation (12). GCs also enable the lactogenic effect of prolactin by inducing the expression of prolactin receptors in the mammary gland (12). The second stage of lactogenesis (Lactogenesis II or secretory activation) occurs up to 4 days after birth, following the decline in progesterone levels, marked by copious milk production (43).

The concentration of cortisone in HM was up to 8 times higher than that of cortisol, consistent with previous studies (13, 15, 16, 24). The cortisol-to-cortisone ratio, usually taken to

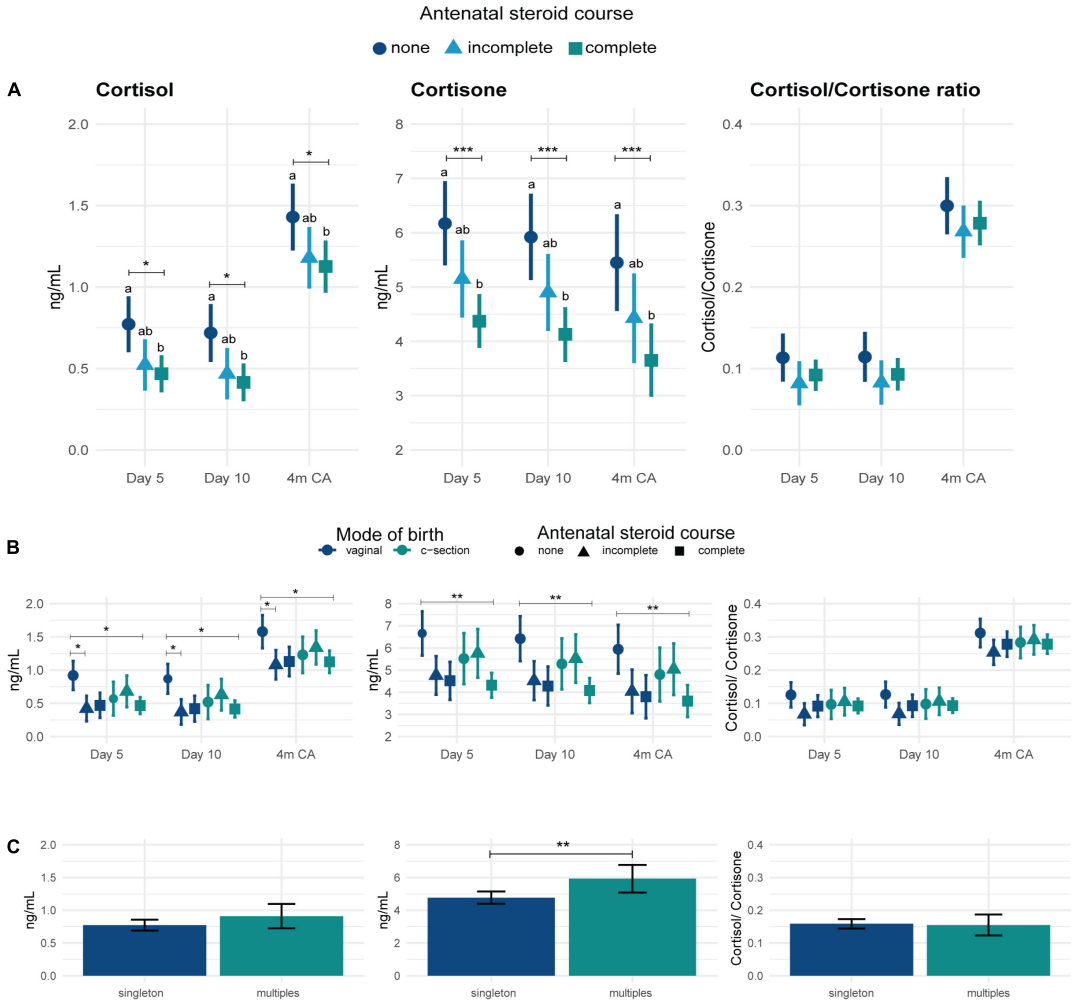

FIGURE 3
Concentrations of cortisol, cortisone and cortisol-to-cortisone ratio in human milk. **(A)** After different antenatal steroid courses and times of sample collection, **(B)** after different modes of birth (dark blue: vaginal birth; green: c-section) and antenatal steroid courses (circle: none; triangle: incomplete; square: complete) and time of sample collection, and **(C)** after singleton or multiple pregnancy. Data are mean concentration (ng/ml) and standard error. Statistically significant differences are represented by different letters above the symbols. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 4m CA: 4 months corrected age.

reflect 11β-HSD2 activity (44), was low throughout lactation. Thus, it seems likely that 11β-HSD2 may be active in the mammary gland to prevent inappropriately high maternal cortisol concentrations transferring into HM, thereby protecting the infant from exposure to excess glucocorticoid. An alternative possibility is that transferal of maternal cortisone into HM is greater than that for cortisol. The role of 11β-HSD2 in protecting the renal mineralocorticoid receptor from activation by cortisol and its role in the placenta in protecting the fetus from exposure to high levels of maternal cortisol have been well-described (44–47). Excessive maternal cortisol concentration during pregnancy due to maternal stress is linked to lower birth weight (48, 49), shorter gestation (49) and impaired neonatal stress regulation (50). Reduced placental 11β-HSD2 activity, measured in cord blood at birth, has been associated with high systolic blood pressure at 3 years of age (51). Exposure to antenatal synthetic GCs, which cross the placental barrier without inactivation by 11β-HSD2, may lead to insulin resistance in adulthood (52). 11β-HSD2 localization in other mineralocorticoid-responsive tissues, including salivary and sweat glands, epithelial tissue (44), and in ductal epithelial mammary cells of non-lactating females (53), also has been reported. Nevertheless, more studies are needed to elucidate the expression and activity of mammary 11β-HSD2 during lactation.

Cortisol and cortisone were highly correlated, with strength of correlation decreasing throughout lactation possibly due to reduction in sample size at 4 months CA. The concentrations of cortisol detected in our study are lower than reported in previous studies (24, 27, 28) including one on preterm HM

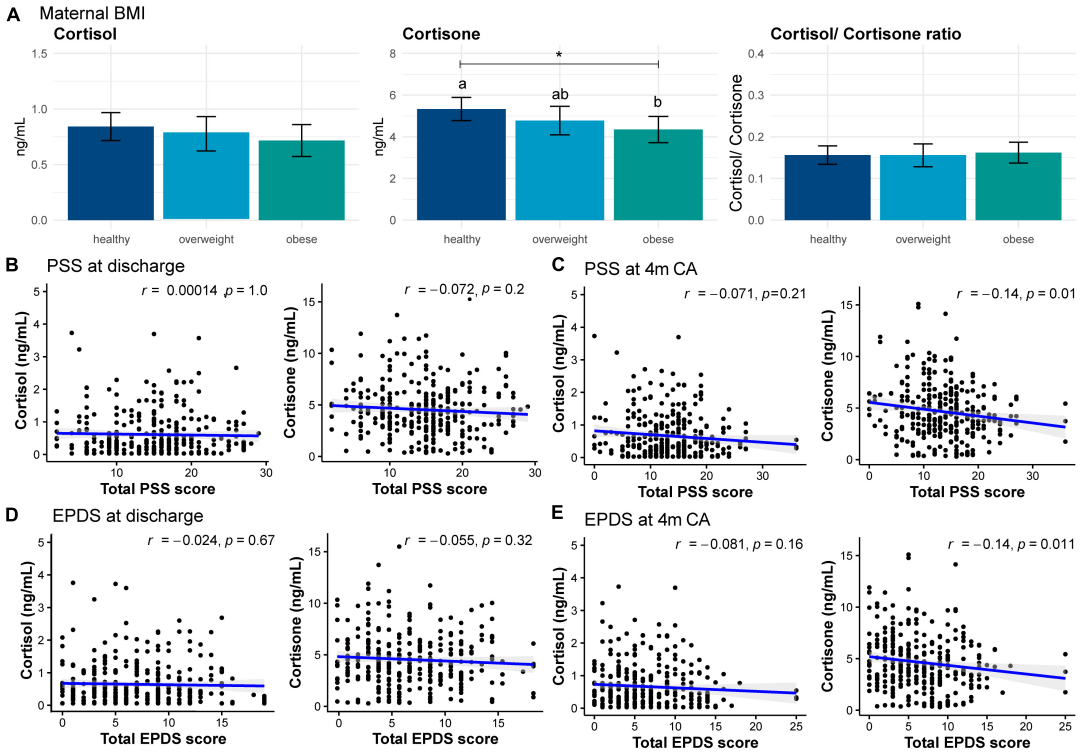

FIGURE 4
Cortisol and cortisone concentrations in human milk in women with different. **(A)** Body mass index (BMI), **(B)** Total Perceived Stress Score (PSS) at discharge, **(C)** Total PSS at 4 months, **(D)** Total Edinburgh Postnatal Depression Scale (EPDS) at discharge, and **(E)** Total EPDS at 4 months (4 m). Blue line and shaded area indicate regression line and 95% confidence interval, respectively. Data are mean concentration (ng/ml) and standard error. Statistically significant differences are represented by different letters above the bars. *$p < 0.05$.

(21). Possible reasons include high exposure to ANS in our cohort, and differences in study population or methodology, since most studies were conducted in Caucasian (15, 21, 27, 28) or European (13, 16, 24, 26) mothers, included mothers of full term infants (15, 16, 22, 24), employed cross-sectional analysis of mature HM (15, 24), and used different analytical methods (27). The concentrations we report are, however, similar to one study involving extremely and very preterm infants (13). We previously have demonstrated that medium chain fatty acids and metabolic hormones in preterm HM differed by maternal ethnicity (54, 55) but it is unclear if ethnicity influences the concentration of GCs in HM. Despite the multi-ethnic population, our study is not fully representative of the general population of woman giving birth in New Zealand (56), with Māori and Caucasian mothers being under-represented and Asian and Pacific mothers over-represented compared to the national birthing population.

Administration of ANS is widely recommended for woman at risk of preterm birth to reduce neonatal respiratory morbidity and mortality risks (10) and the majority of mothers in our study received some ANS. Our findings suggest that receipt of even a single dose of ANS given less than 24 h before birth is associated with lower GCs concentration in HM through to 4 months, even among mothers who birth vaginally. Possible explanations include suppression of the maternal HPA axis in response to the high levels of exogenous glucocorticoid (57), disruption of the hormonal switches that lead to secretory activation (12, 41) or other underlying health complication leading to ANS administration due to risk of preterm birth, such as chorioamnionitis or premature rupture of membranes. It is unlikely that maternal HPA axis suppression following administration of ANS persists for 4 months postpartum, implying a more profound effect of ANS on mammary gland development and lactation, although we do not have maternal plasma cortisol concentrations with which to compare the HM concentrations.

Along with the rise in maternal GCs levels in late gestation (7), studies in both rats and cattle demonstrate that GC receptors in the mammary gland increase during pregnancy, peak around parturition and return to pregnancy levels during lactation (12, 42). Studies in lactating rats suggest that administration of synthetic GCs (dexamethasone 21-acetate) down-regulates GC receptors in mammary gland cytosol, with receptor binding affinity remaining low even 48 h after synthetic GC withdrawal (58). In humans, Henderson et al. reported reduced milk volume among mothers of preterm infants who received antenatal

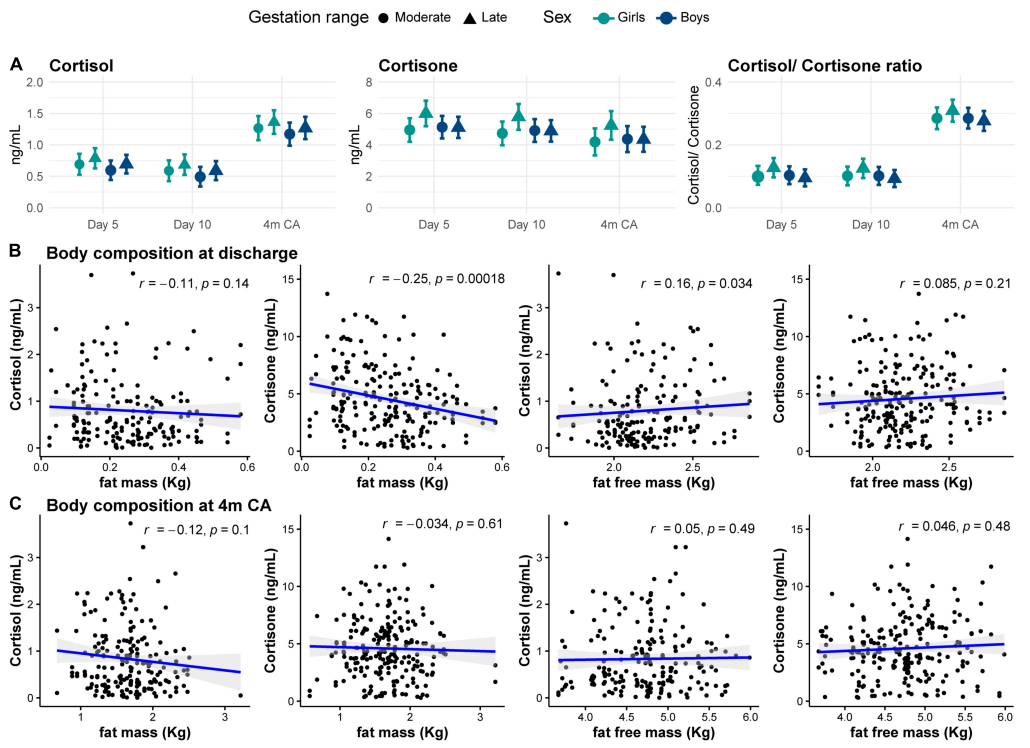

FIGURE 5
(A) Cortisol and cortisone concentrations in human milk for moderate (circles) and late (triangles) preterm girls (green) and boys (blue) at each collection age. Data are mean concentration (ng/ml) and standard error. Correlation between human milk cortisol and cortisone concentrations and body composition **(B)** at hospital discharge and **(C)** at 4 months corrected age (4m CA). Blue line and shaded area indicate regression line and 95% confidence intervals.

steroids 3–9 days before birth compared with mothers giving birth up to 2 days after ANS treatment, but markers of secretory activation such as lactose and citrate did not differ between study groups (59). Since timing of ANS administration often coincides with Lactogenesis I, it is possible that down-regulation of mammary GC receptors leads to low GC concentration in HM; however, the length of suppression observed in our study is surprising and we are unaware of other human studies investigating the long-term effects of ANS on lactogenesis.

Altered HPA axis activity has been associated with several pathological conditions, including hypertension, dyslipidemia, and obesity (6). On one hand, acute stress often induces short-term rises in plasma cortisol concentrations (6, 9). In contrast, chronic stress and prolonged exposure to GCs may induce structural changes to the HPA axis, leading to suppression of HPA activity and blunted cortisol reactivity, often referred to as "wear and tear" effect or allostasis (60), as reported in chronic depression and anxiety (61) and obesity (62). Our finding that maternal obesity is associated with lower GC concentrations in HM is consistent with data from a large Finnish cohort (24). The association between higher scores in both stress and postnatal depression and lower GCs in HM is less clear and contrary to the general assumption of positive relationship between stress and cortisol. Psychosocial distress at 3 months postpartum as a composite of maternal stress, anxiety and postpartum depression symptoms has been reported to be positively associated with cortisol concentrations in HM (22). Whereas Lindberg et al. identified a negative correlation between HM cortisol concentrations and maternal anxiety, but not postpartum depression symptoms, 3 months after birth (26), the large Finnish cohort found no association between maternal distress and GCs in HM at 3 months postpartum (24). Since both PSS and EPDS are indicative of mid- to long-term stress and depressive symptoms, they likely reflect chronic rather than acute stress and this may explain the association with lower maternal GC concentrations. Of note, none of these studies focused on mothers of preterm infants who often experience stressful situations in the postnatal period.

We did not find significant associations between GC concentrations in HM and size at birth, infant growth or body composition, apart from weak correlations between cortisone and birth head circumference *z*-score, cortisol and infant fat free mass, and cortisone and fat mass. One study previously has shown that higher HM cortisol concentration at 3 months was predictive of lower body mass index percentile during first 2 years of life (27). However, others reported positive

associations between HM cortisol concentrations and infant fat mass in the first year (28). Therefore, the effect of HM GCs on postnatal growth and body composition remains inconclusive.

Studies on longitudinal changes of GC concentrations in HM have yield conflicting results. While some have reported that these remain unchanged over time (21, 28), others suggest an increase in cortisol concentrations as lactation progresses (22). It is possible that lower GC concentrations that we observed in early lactation may be due to reduced maternal HPA axis activity following birth and cessation of placental corticotrophin-releasing hormone (CRH) secretion (7), since both of our early lactation samples fell within first 10 postnatal days. Effects of lactation stage on HM composition, including of hormones such as GCs, may have important implications for HM donation and nutrition of preterm infants. GCs are preserved during pasteurisation (63) and donor HM may have different profile of hormones when compared to mothers' own milk, especially when circadian cycles are not considered (5, 25). Thus, a hormonal mis-match may occur when infants are not breastfeeding or not receiving HM expressed at similar times to feeding, with unclear implications for infant development and metabolic programming.

Strengths and limitations

Our study has several strengths, including the large cohort of moderate to late preterm infants and standardized HM collection protocol, which ensured that majority of samples obtained were collected from full breast expression in the morning. The superiority of LC-MS for quantification of HM GCs (64) and the standardized growth and body composition assessments permitted robust investigation of associations between HM GCs and postnatal growth. Although detailed nutritional intake data were collected as part of the DIAMOND trial, an estimation of GC intake was not possible since most moderate and late preterm infants are also breastfed during their hospital stay and HM samples were limited to a morning sample on postnatal day 5 and day 10, which would not accurately reflect the diurnal GC variation in HM and the actual intake by the infant. Further, as this study is a secondary cohort analysis, with sample size determined by the participants of the main trial who provided HM samples, it is possible that unknown factors not investigated (such as pre-pregnancy BMI or maternal body composition) could have confounded the associations identified, and thus caution is warranted when extrapolating current findings to another population.

Future directions

Further research is needed to elucidate the effect of ANS on lactogenesis and potential impacts on the preterm infant. Given that preterm infants often receive HM at times mis-matched to collection times, the potential short- and long-term effects of feeding preterm infants HM with different hormone levels (hormonal mis-match), such as HM expressed at different time of the day or donor HM (often mature HM pooled from many donors) requires further research. Assessment of circulating GC concentrations in both infant and mother using saliva and/or plasma samples in parallel with HM collection will help to investigate further the mother-milk-infant communication *via* HM hormones. Furthermore, more animal and *in vitro* studies are required to characterize the expression and activity of mammary 11β-HSD2 during lactation.

Conclusion

The mammary gland appears to protect the infant from cortisol through inactivation into cortisone. GCs in HM were inversely correlated with maternal stress, postnatal depression and BMI, and very weakly correlated with infant size at birth, in-hospital growth, and body composition at hospital discharge. ANS may have lasting effects on the concentration of GCs in HM and, given that most mothers at risk of preterm labor are exposed to ANS, more research is needed to understand the implications of ANS on lactogenesis and whether HM GCs can influence HPA axis function, growth and development of moderate and late preterm infants.

Data availability statement

The raw de-identified individual participant data supporting the conclusions of this article will be made available by the authors (including data dictionaries) upon reasonable request and after review by the Data Access Committee of the Liggins Institute. Proposals should be submitted to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by New Zealand Health and Disability Ethics Committee (number: 16/NTA/90). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

MM assisted with sample collection, carried out statistical analysis, interpreted the results, and drafted the manuscript.

TA designed the randomized controlled trial, provided funding for sample and data collection, and contributed to the manuscript development. MV contributed to interpretation of results and the manuscript development. JH designed the randomized controlled trial and contributed to manuscript development. LG designed breastmilk collection protocol and contributed to the manuscript development. FB designed the randomized controlled trial, provided funding for sample and data collection, contributed to interpretation of results, and the manuscript development. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Extracellular vesicle miRNAs in breast milk of obese mothers

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Background: Breast milk has abundant extracellular vesicles (EVs) containing various biological molecules (cargo), including miRNAs. EVs are not degraded in the gastrointestinal system and circulation; thus, breast milk EVs (bEVs) are expected to interact with other organs in breastfed infants and modify the gene expression of recipient cells using miRNAs. Maternal pre-pregnancy BMI is a critical factor influencing the composition of breast milk. Thus, in mothers with obesity, miRNAs in bEVs can be altered, which might be associated with adverse health outcomes in infants. In this study, we examined 798 miRNAs to determine which miRNAs are altered in the bEVs of mothers with obesity and their potential impact on breastfed infants.

Methods: We recruited healthy nursing mothers who were either of normal weight (BMI $<$ 25) or with obesity (BMI $\geq$ 30) based on their pre-pregnancy BMI, and delivered a singleton baby in the prior 6 months. EVs were isolated from breast milk with ultracentrifugation. bEV characteristics were examined by flow cytometry and fluorescence imaging of EV markers. A total of 798 miRNAs were screened using a NanoString human miRNA panel to find differentially expressed miRNAs in bEVs of mothers with obesity compared to mothers of normal weight.

Results: We included 65 nursing mothers: 47 of normal weight and 18 with obesity based on pre-pregnancy BMI. After bEV isolation, we confirmed the expression of various EV markers. Out of 37 EV markers, CD326 (EpCaM) was the most highly expressed in bEVs. The most abundant miRNAs in bEVs include *miR-30b-5p, miR-4454, miR-494-3p*, and *let-7 miRNAs*. Target genes of the top 10 miRNAs were associated with cancer, prolactin pathway, EGFR, ErbB, and FoxO signaling pathway. In bEVs of mothers with obesity, 19 miRNAs were differentially expressed (adjusted $p < 0.05$ cut-off), which include *miR-575, miR-630, miR-642a-3p*, and *miR-652-5p*. These miRNAs and their target genes were associated with neurological diseases and psychological disorders.

Conclusion: In this study, we characterized bEVs and demonstrated altered miRNAs in bEVs of mothers with obesity and identified the pathways of their potential target genes. Our findings will provide insight for future studies investigating the role of bEVs in breastfed infants.

KEYWORDS

breast milk, maternal obesity, extracellular vesicles, exosomes, miRNAs

Introduction

Extracellular vesicles (EVs) are nanosized particles (30–150 nm in diameter) released from cells (1). EVs encapsulate biological molecules (cargos) of donor cells during the biogenesis of EVs, which include non-coding RNAs, lipids, and proteins. Because EVs are surrounded by a lipid bilayer, they are not degraded in the gastrointestinal system and circulation. Thus, EVs can interact with other cells and modify the gene expression of recipient cells by transferring internal cargo molecules such as miRNAs (2). Milk of mammals has abundant EVs (3, 4). After oral administration, milk EVs are distributed in the intestine and other distal organs, including the liver, spleen, heart, and brain (4, 5). Thus, human milk EVs and EV cargos are considered to interact with multiple organs and play a significant role in the growth and development of breastfed infants. Furthermore, milk EVs from humans and other mammals are emerging as therapeutics (6, 7). In several pathological conditions such as ulcerative enterocolitis and cancers, it has been demonstrated that milk EVs enhance immunity and suppress inflammation (8, 9). The therapeutic characteristic of milk EVs may be associated with the miRNA profiles found in milk EVs (10). However, the signaling mechanism associated with miRNAs of milk EVs and the major role players among miRNAs in EVs have not been fully elucidated. Therefore, understanding breast milk EVs (bEVs) and their miRNA profiles is critical not only to increasing our knowledge about bEV dynamics in infants but also to developing new therapeutics using bEVs.

The population of women with obesity of childbearing age has significantly increased in the US (11, 12). Increased maternal BMI critically influences the component of breast milk. In mothers with overweight and obesity, lipids, metabolites, and immunological components of breast milk were dysregulated, which are associated with adverse impacts on infants' physical/mental health and development (13–15). miRNAs are also altered in mothers with overweight and obesity. Especially altered miRNAs encapsulated by EVs can cross biological barriers and be internalized into other distal organs without degradation (10, 16). Thus, it is critical to understand which miRNAs are altered in mothers with obesity, and their potential impact on breastfed infants. Recently, a few selected miRNAs associated with adipogenesis and glucose metabolism were tested in bEVs from mothers with overweight and obesity (17). *miR-148a* and *miR-30b* were downregulated in mothers with overweight and obesity, which are related to the body composition of infants. However, this study examined only a few miRNAs, which could not provide a broader view of miRNAs in bEVs that are altered in maternal obesity and their potential impacts. In this study, we compared the expression level of 798 biologically relevant miRNAs in bEV between mothers with obesity and mothers of normal weight and analyzed enriched pathways of differentially expressed miRNAs found in mothers with obesity. Our findings provide a comprehensive understanding of the altered bEV miRNAs in maternal obesity and their potential impacts on breastfed infants.

Materials and methods

Participants

We recruited healthy nursing mothers who were ≥18 years old and had delivered a full-term (37–40 weeks) singleton newborn within the prior 6 months. Mothers whose pre-pregnancy BMI was either <25 (normal weight) or ≥30 (obesity) were included in this analysis. Mothers with chronic conditions that might affect body weight changes, such as chronic diabetes or thyroid diseases, were excluded. After electronic informed consent was obtained, a breast milk sample collection kit was mailed to the participant's home with detailed instructions. After 1 h of fasting, participants collected 50 ml of breast milk and froze it in their home freezer. Within 48 h of sample collection, samples were returned to the lab and stored at −80°C until use. Participants also completed an online demographic survey and the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool (18). All procedures were followed under an approved IRB protocol (IRB #202005237).

Breast milk extracellular vesicle isolation and characterization

Breast milk EVs were isolated using ultracentrifugation. First, frozen breast milk was thawed at 4°C overnight. Breast milk (15 ml) was centrifuged at 3,000 $g \times 3$ to remove bulk fat. The remaining supernatant was then centrifuged at

12,000 *g* for 40 min to remove the remaining fat and cell debris. After filtering with a 0.22-μm syringe filter, samples were centrifuged at 100,000 *g* for 2 h to pellet EVs. The EV pellet was resuspended in 300 μl sterile PBS and rotated overnight at 4°C. EV characteristics were tested using the ExoView R100 platform (NanoView Bioscience, MA, United States), which allowed us to measure the expression of general EV markers, including CD81, CD63, and CD9, using fluorescence antibodies. The number of particles labeled by fluorescence antibodies was counted, and their size was measured. In addition, we tested surface epitopes of bEVs using the MACSPlex Exosome Kit (Miltenyi Biotec, Germany), which can detect up to 37 surface epitopes of EVs. Flow cytometric analysis was performed with a MACSQuant Analyzer 10 flow cytometer equipped with 405, 488, and 638-nm lasers and a built-in 96-well plate reader (Miltenyi Biotec, Germany).

The expression level of CD326 and TSG101 was examined using a western blot. The protein level of EVs was measured using a Qubit protein assay kit (ThermoFisher Scientific, MA, United States), and equal amounts of protein were loaded on SDS-PAGE gel. Proteins were transferred to a PVDF membrane and blocked with a 5% skim milk solution for 1 h. The membrane was then incubated with a CD326 antibody (Santa Cruz Biotechnology, CA, United States) and TSG101 antibody (ThermoFisher Scientific, MA, United States) overnight at 4°C. Signals were detected with an HRP-conjugated antibody (Santa Cruz Biotechnology, CA, United States) using the ECL-Pico system (ThermoFisher Scientific, MA, United States).

miRNA profiling with NanoString miRNA assay

After NanoString assay was performed, data were obtained as "RCC" format files. These were imported into R with the *readRcc* function from the NanoString QCPro package (19). We followed the "Remove-Unwanted-Variation-genes" (RUVg) normalization procedure for NSolver data outlined in Love et al. (20). RUVg normalization uses negative control genes (RUVg) to remove unwanted technical and batch variation in the data, and has been shown to be superior to the NSolver-provided normalization procedure (21). All samples passed nSolver QC checks ("imaging," "binding density," "linearity of positive controls," and "Limit of detection") and were included in the analysis. Next, the five housekeeping genes on the panel were assessed for correlation with sample BMI; no statistically significant correlation was detected for any of the five genes. We also check the technical batches for the number of genes below the limit of detection (LOD). Batch 4 (of 9) did show a higher proportion of endogenous genes below the LOD (data not shown), but we chose to retain the samples in the analysis and remove the unwanted variation through the normalization procedure.

Remove-Unwanted-Variation-genes normalization was applied to the raw NSolver data. Normalized data were visualized with relative log expression (RLE) and principal component analysis (PCA) plots for different values of "k" (k = 1,2,3). RLE and PCA plots on RUVg normalized data showed clear removal of systematic technical effects owing to batch in the data, particularly at k = 2 and k = 3 (data not shown). Thus, RUVg-normalized data with k = 2 was chosen for downstream differential expression analysis. A DESeq2 object was created from the raw data using *DESeqDataSetFromMatrix* with design conditioned on the BMI factor variable, the postpartum date quartile, and the RUVg (k = 2) "W1" and "W2" correction factors learned for technical batch effects. DE analysis proceeded according to best practices as described in the DESeq2 vignette. The R scripts used for exploratory analysis and final DE analysis are available on Github (https://github.com/mchimenti/project_nanostring_miRNA_breastmilk_jan2022/blob/115a6577559186a02b466d3cff1fa38d8258c782/nanostring_deseq2_analysis_cho_jan2022.Rmd and https://github.com/mchimenti/project_nanostring_miRNA_breastmilk_jan2022/blob/115a6577559186a02b466d3cff1fa38d8258c782/nanostring_deseq2_analysis_Version2_cho_Mar2022.Rmd, respectively).

Statistical analysis

Demographics and the expression of EV markers between groups were compared by the Mann-Whitney U test with a statistical threshold of p = 0.05 using SPSS 27 (IBM, NY, United States). Ingenuity Pathway Analysis (Qiagen, Germany) was used to analyze network interactions among differentially expressed miRNAs and their target genes. KEGG pathway enrichment analysis was performed using DAVID online tools[1] (22, 23). Using a Benjamini–Hochberg method, the false discovery rate of the pathway analysis was calculated in the KEGG enrichment pathway analysis. All plots were generated using GraphPad Prism 7 (GraphPad Software, Inc., CA, United States).

Results

We analyzed 65 human breast milk samples from healthy nursing mothers who had delivered a baby within the prior 6 months. The mean age of participants was 32.7 years, and 92% of the participants were White. We included mothers whose pre-pregnancy BMI was <25 (normal weight, n = 47) and ≥30 (obesity, n = 18). Breast milk samples were collected an average of 66 days postpartum. Detailed demographic characteristics are

1 https://david.ncifcrf.gov/

described in Table 1, and metadata is in the Supplementary Document.

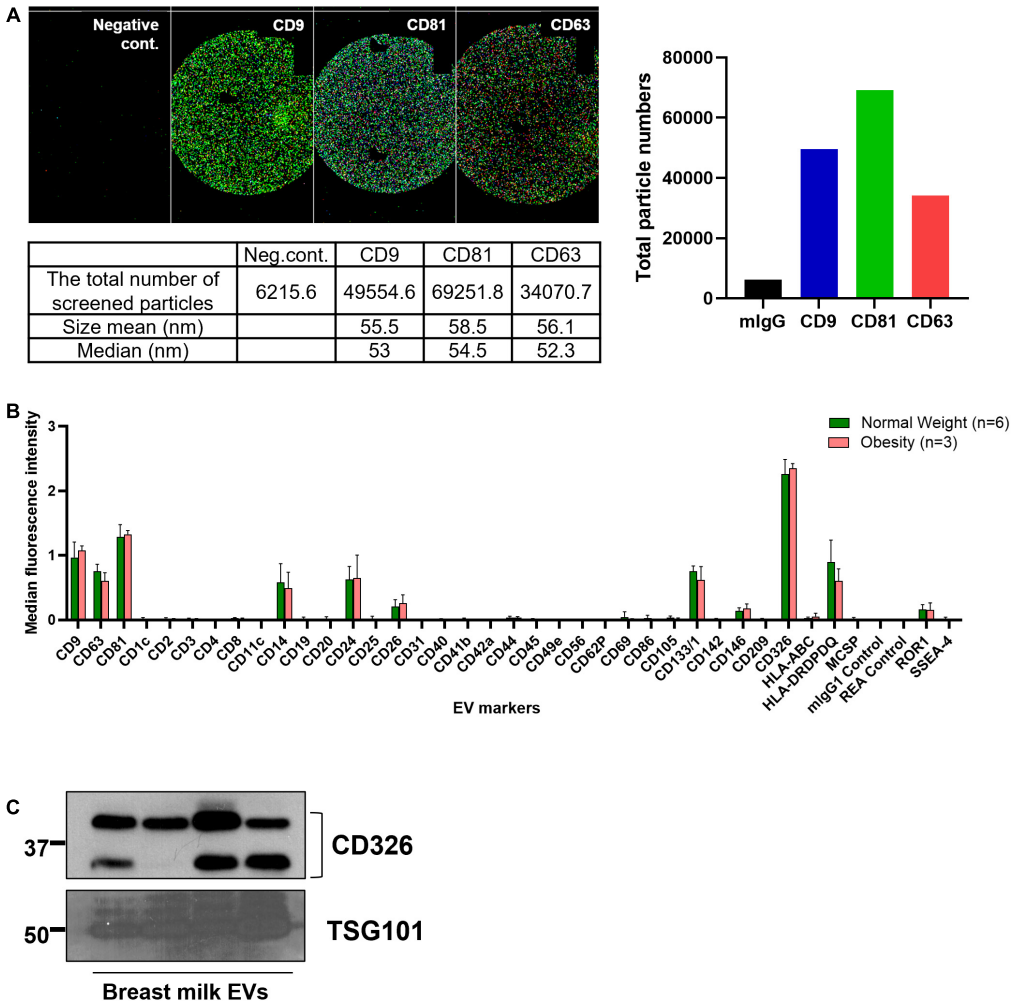
The presence of EVs was confirmed by the expression of EV markers, including CD9, CD63, CD81, and TSG101, and by the size of the particles (Figure 1A). According to the analysis of surface epitopes, CD14, CD24, CD133/1, CD326, and HLA-DRDPDQ were highly expressed in bEVs, in addition to the general EV markers CD9, CD63, and CD81. Out of 37 markers analyzed, CD326 was the most abundant EV marker expressed in bEVs. The expression level of CD326 was also confirmed with western blotting (Figures 1B,C).

Next, we examined the expression level of 798 miRNAs and analyzed the most abundant miRNAs in bEVs. All 798 miRNAs were detected and included in the differentially expressed miRNA analysis. The top 10 most abundant miRNAs are listed in Figure 2, including *miR-30b-5p, miR-4454 + miR-7975, miR-494-3p*, and *let-7a-5p*. Enrichment pathways analysis with target genes of top 10 bEV miRNAs demonstrated that cancer, epidermal growth factor receptor (EGFR), prolactin signaling pathway, erythroblastic leukemia viral oncogene homologue (ErbB), and forkhead box, class O (FoxO) signaling pathways are enriched (Figure 2B). The top 10 most abundant bEV miRNAs were the same between groups with normal weight and obesity, except for one miRNA (Supplementary Figure 1). The expression level of the top 10 most abundant miRNAs was compared between groups, demonstrating that there was no significant difference (Supplementary Figure 2).

Differentially expressed miRNAs in bEVs of mothers with obesity were analyzed after controlling for postpartum dates when samples were collected. Nineteen miRNAs were significantly differentially expressed in bEVs of mothers with obesity, including *miR-575*, *miR-630, miR-642a-3p*, and *miR-652-5p* (Figure 3A, B). The median and interquartile range (IQR) of the normalized counts of these miRNAs are shown in

TABLE 1 Subject demographics.

	All participants	Lean (pre-pregnancy BMI < 25, $n = 47$)	Obese (pre-pregnancy BMI≥ 30, $n = 18$)	p value
Age (years)	32.7 ± 3.7	33.0 ± 3.8	32.1 ± 3.4	0.396
Race				
White	60 (92.3%)	47 (91.5%)	18 (94.4%)	0.385
Asian	2 (3.1%)	2 (4.3%)	0	
African American	1 (1.5%)	0	1 (5.6%)	
Multiracial	1 (1.5%)	1 (2.1%)	0	
Unknown	1 (1.5%)	1 (2.1%)	0	
Pre-pregnancy BMI	25.2 ± 5.6	22.0 ± 1.9	33.7 ± 2.5	<0.001
Current BMI	26.8 ± 5.1	24.1 ± 2.8	33.8 ± 2.4	<0.001
Body weight changes during pregnancy (Ibs)	29.1 ± 9.5	31.8 ± 8.4	22.2 ± 9.1	<0.001
Postpartum sample collection (days)	65.9 ± 49.0	65.9 ± 46.0	65.8 ± 57.6	0.363
Parity				
1	14 (21.2%)	10 (21.7%)	4 (22.2%)	0.555
2	30 (45.5%)	23 (50.0%)	7 (38.9%)	
3	16 (24.2%)	11 (23.9%)	5 (27.8%)	
4 or more	4 (6.2%)	2 (4.3%)	2 (11.1%)	
Gestation period (weeks)	39.1 ± 1.2	39.0 ± 1.3	39.1 ± 0.8	0.606
Delivery mode				
C-section	9 (13.8%)	6 (12.8%)	3 (16.7%)	0.767
Vaginal delivery	56 (86.2%)	41 (87.2%)	15 (83.3%)	
Nursing methods				
Direct breastfeeding	57 (87.7%)	43 (91.5%)	14 (77.8%)	0.132
Bottled feeding	35 (53.8%)	22 (46.8%)	13 (72.2%)	0.066
Formula feeding	10 (15.4%)	6 (12.8%)	4 (22.2%)	0.344
Pregnancy complication				
Pre-eclampsia	7.7% ($n = 5$)	1 (2.2%)	4 (22.2%)	0.007
Gestational diabetes	4.6% ($n = 3$)	1 (2.2%)	2 (11.1%)	0.128
Average intake per day (cal)	2,438.5 ± 773.4	2435.7 ± 760.9	2446.1 ± 830.4	0.964



	Neg.cont.	CD9	CD81	CD63
The total number of screened particles	6215.6	49554.6	69251.8	34070.7
Size mean (nm)		55.5	58.5	56.1
Median (nm)		53	54.5	52.3

FIGURE 1
Characteristics of breast milk EVs (bEVs). **(A)** Particles expressing three EV markers (CD9, CD81, and CD63) were examined using NanoView R100 (n = 8). The representative images show the colocalization of these three EV markers; CD9 in dark blue; CD81 in green; and CD63 in red. mIgG is a negative control. The size range of EVs expressing these markers is 50–60 nm. **(B)** The median fluorescence intensity of EV markers was calculated after background correction with negative controls,and the intensity was compared between groups. **(C)** The expression level of CD326 and TSG101 in bEVs was confirmed with western blotting (n = 4).

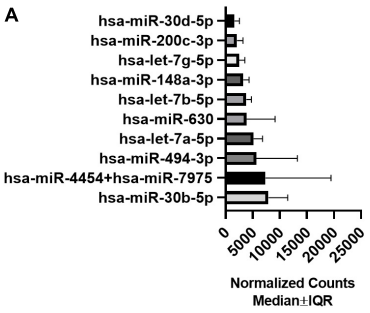
Table 2. The top network of these miRNAs and their predicted target genes was associated with neurological diseases and psychological disorders (Figure 3C). In this network, the hub gene was TP53, which is predicted to be activated (Figure 3D).

Discussion

miRNAs of bEVs are considered to play an important role in the growth and development of infants. Maternal BMI influences miRNA profiles; thus, knowing about altered miRNAs of bEVs in maternal obesity is critical for better understanding the role of breast milk in infants (10). Furthermore, it has recently been suggested that bEVs have therapeutic properties for multiple pathological conditions, including intestinal diseases and cancers (7). Because major EV cargos are miRNAs, miRNAs are considered major role players in the therapeutic effect. Thus, profiling miRNAs in bEVs will help figure out the potential signaling pathways of therapeutic effect.

Mammary epithelial cells are thought to be the most dominant cells contributing to breast milk production (24, 25). We found that bEVs expressing CD326 (EpCAM) are the most abundant in breast milk. CD326 is an epithelial cell adhesion molecule used as a marker for epithelial cells in body fluids (26). The ectodomain of CD326, EpEX, is a ligand of EGFR, which is highly expressed in the mammalian intestinal epithelium (27, 28). EGFR is a key signaling molecule involved in intestinal cell growth, repair, and migration (29). Although non-specific EV uptake is generally shared in most cell types, CD326

A



B

Term	Genes	%	p-value	Benjamini
MicroRNAs in cancer	22	4.8	4.30E-06	1.10E-03
EGFR tyrosine kinase inhibitor resistance	10	2.2	5.60E-05	6.90E-03
Prolactin signaling pathway	9	2	1.40E-04	1.20E-02
ErbB signaling pathway	9	2	5.50E-04	3.00E-02
FoxO signaling pathway	11	2.4	6.20E-04	3.00E-02

FIGURE 2
Top 10 most abundant miRNAs in bEVs and enriched pathways of target genes. **(A)** The top 10 most abundant miRNAs were identified using normalized median counts (n = 65). **(B)** Enriched pathways of target genes of the top 10 miRNAs of bEVs are demonstrated.

expression on bEVs might enhance the interaction between bEVs and intestinal epithelial cells of infants. In addition, it was recently revealed that CD326 regulates intestinal epithelial integrity and stem cells. CD326 enhanced the growth of intestinal epithelial cell organoids and spheroids (30). Multiple studies have demonstrated that milk EVs improve pathological intestinal conditions (7, 8, 31), and miRNAs are expected to play a critical role in the therapeutic impact of milk EVs. However, considering the high expression of CD326 and the role of CD326 in the intestine, the contribution of CD326 to the therapeutic impact of milk EVs may need to be examined further.

In order to understand the content of bEVs, several studies have profiled miRNAs of bEVs. There are differences across the studies in the characteristics of mothers, breast milk collection time, EV isolation methods, and miRNA profiling platforms, therefore, the results of studies are inconsistent. Despite that, many studies have shown that *miR-148a-3p, let-7 miRNAs,* and *miR-200c-3p* are abundantly expressed in bEVs, which are associated with promoting immune function and inflammatory regulation (32–35). Meanwhile, another study shows completely different abundant miRNAs, such as *miR-4271, miR-3197*, and *miR-2861* (36). These are involved in the estrogen signaling pathway, ECM-receptor pathway, axon guidance, and various cancers. Our study found that *miR-200c-3p, miR-148a-3p*, and multiple *let-7 miRNAs* including *let-7a-5p, let-7b-5p, and let-7g-5p* are abundant in bEVs as many other studies have demonstrated. However, we also found different miRNAs, for example, *miR-4454* + *miR-7975*. These two miRNAs were grouped together in this platform because the mature sequence of *miR-7975* differs by only one base from *miR-4454* (37). Previous reports showed that *miR-4454* promotes inflammation or cancer invasion and migration (38, 39). However, the role of either *miR-4454* or *miR-7975* in breast milk for infants' health and development is relatively unknown. One study reported that *miR-4454* was deregulated in mothers of premature infants compared to mothers of term infants, however, the role of this miRNA was not elucidated (40). In order to fully understand the content of bEVs and the role of bEVs on infants' health, more studies with controlled sample collection methods and profiling platforms would be needed.

The top 10 most abundant miRNAs identified in bEVs are associated with cancers and prolactin signaling pathways, which might be related to mammary epithelial cells. As shown in **Figure 1**, the majority of bEVs originated from mammary epithelial cells. Most human breast cancers arise from the epithelial cells (41), and prolactin stimulates mammary epithelial cells to synthesize milk components (42). Besides that, EGFR, ErbB, and FoxO signaling pathways were identified as enriched patyways in miRNAs of bEVs. EGFR and ErbB2 signaling lead to downstream activation of FoxO3, which regulates gene expression affecting apoptosis, cell-cycle control, glucose metabolism, and oxidative stress (43). Activation of these molecules is involved in various disease mechanisms, including cancers, type 2 diabetes, and cardiovascular diseases (44, 45). Abundant miRNAs in bEVs might inhibit the role of their target genes in the recipient cells, and resulting in a beneficial effect (46). In fact, previous studies demonstrated that abundant miRNAs in bEVs, including *miR-30b-5p, miR-494-3p*, *miR-148a-3p*, and the *let-7 miRNAs*, suppress tumor progression and development and reduce inflammation (47–49). In intestinal epithelial cells, bEVs inhibited the TLR4/NFκB and NLR4 inflammasome pathways and reduced inflammation (8). EGFR and TLR4 pathways are interrelated and target transcription factors such as NFκB (50, 51). Further studies to define the precise downstream pathways modulated by bEVs will deepen our understanding of the beneficial role of bEVs.

Previously, studies have shown altered breast milk miRNAs in increased maternal pre-pregnancy BMI (36). The expression patterns of miRNAs related to leptin and adiponectin, including *miR-222*, *miR-103*, *miR-17*, *miR-let-7a*, and *miR-let-7c,* were different in mothers with overweight and obesity compared to those in mothers with normal weight (52). In bEVs of mothers with overweight and obesity, *miR-148a* and *miR-30b* related to adipogenesis and glucose metabolism, were downregulated compared to mothers with normal weight (17). Interestingly, the expression level of these miRNAs was

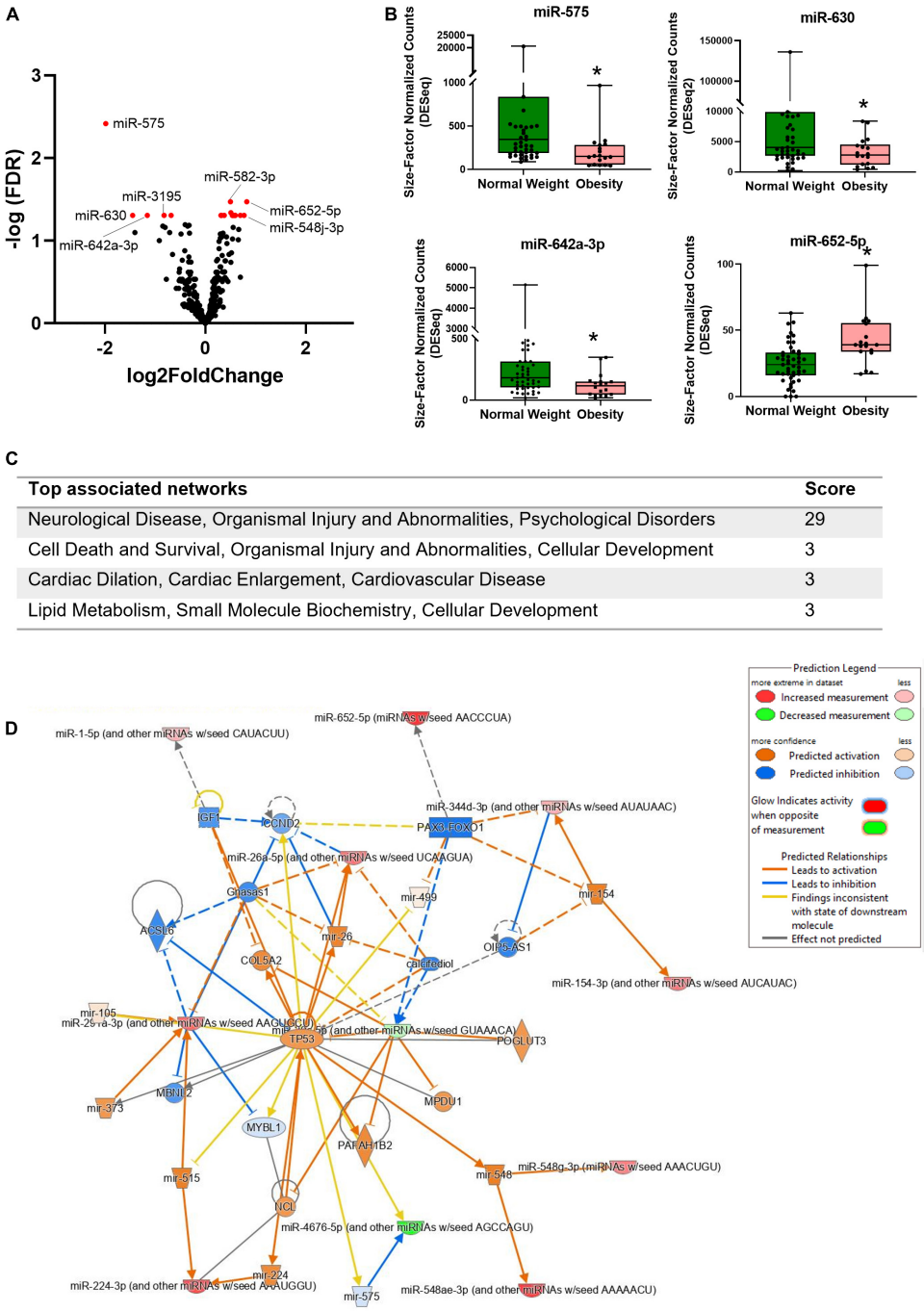


FIGURE 3
Differentially expressed miRNAs in bEVs of mothers with obesity. **(A)** Volcano plot shows the distribution of miRNAs in bEVs with fold changes and *p* values. miRNAs in red represent deregulated miRNAs with a cut-off of adjusted $p < 0.05$. **(B)** Normalized counts of selected miRNAs are shown between groups. *Represents a statistical significance with adjusted *p* values < 0.05. **(C)** Top associated networks are analyzed using differentially expressed miRNAs and their predicted target genes in bEVs of mothers with obesity. **(D)** The network is the #1 network in panel **(C)**; neurological disease, organismal injury and abnormalities, and psychological disorders. TP53 is a hub gene of this network.

correlated with infants' weight, fat mass, and body fat (%), thus, the authors suggested that these miRNAs might protect infants from obesity and type 2 diabetes later in life. In our study, these miRNAs are abundantly expressed in bEVs, however, their expression levels were not significantly different between groups. Different EV isolation methods, different RNA detection platforms, and different postpartum dates when breast milk was collected might influence this discrepancy. We identified several

TABLE 2 Differentially expressed miRNAs in breast milk EVs of mothers with obesity (pre-pregnancy BMI ≥ 30) compared to mothers with normal weight (pre-pregnancy BMI < 25).

	Normal weight (n = 47)		Obesity (n = 18)		Adjusted p value	log2Fold change
	Median	IQR	Median	IQR		
miR-575	345	646	150.5	224	0.004	−1.983
miR-630	4,038	7236	2796.5	3074	0.049	−1.447
miR-642a-3p	182	211	115.5	103	0.049	−1.154
miR-652-5p	24	17	39	21	0.034	0.825
miR-3195	16	13	11.5	6	0.049	−0.82
miR-548j-3p	1,146	1187	2159	1719	0.049	0.77
miR-522-3p	449	351	634	444	0.049	0.696
miR-30c-5p	161	122	116	43	0.049	−0.679
miR-448	215	137	65	55	0.049	0.595
miR-302b-3p	292	181	451	204	0.049	0.593
miR-219b-3p	130	62	169	86	0.049	0.579
miR-1297	569	356	775.5	392	0.049	0.556
miR-487a-3p	73	47	115.5	70	0.049	0.544
miR-499b-3p	67	39	105	59	0.049	0.539
miR-548g-3p	308	145	403.5	148	0.046	0.509
miR-582-3p	47	25	71	26	0.034	0.502
miR-450b-5p	56	19	66.5	24	0.049	0.378
miR-410-3p	25	7	29.5	12	0.049	0.325
miR-1-5p	56	18	71	15	0.049	0.312

other differentially expressed miRNAs in mothers with obesity, associated with adiposity and obesity, such as *miR-642a*, *miR-30c*, *miR-448*, and *miR-302b* (53, 54). These miRNAs might play a role in either disturbing the metabolism of infants or protecting infants from harmful outcomes.

However, most previous studies that examined altered breast milk components in mothers with obesity demonstrated different results. It is reported that altered fatty acids in mothers with obesity have decreased neuroprotective factors and are associated with the cognition of infants (14, 55). Altered metabolites of breast milk in mothers with overweight and obesity were related to neurodegenerative diseases and neuropsychiatric disorders (56). We also found that altered miRNAs of bEVs in mothers with obesity are involved in neurological and psychological disorders. Interestingly, TP53 was a hub gene of this network and was predicted to be activated. TP53 encodes p53, a well-known cancer suppressor, which plays a critical role in brain development and neural stem cell regulation (57). Previous reports demonstrated that altered regulation of TP53 can lead to various neurological/psychological disorders such as Alzheimer's disease, schizophrenia, and encephalopathy (58–60). Our findings substantiate these previous reports about the potential impact of altered breast milk components in mothers with obesity on the neurological development of breastfed infants (17). A pre-clinical longitudinal study should be performed to elucidate the long-term health outcomes in infants associated with altered miRNA in bEVs of mothers with obesity.

bEVs also provide information about the breasts of nursing mothers. Most bEVs originate from mammary epithelial cells, where most breast cancer arises. Especially, women with obesity are at increased risk of breast cancers, therefore, differentially expressed miRNAs of bEVs in mothers with obesity could be the molecules that either help us to predict breast cancer occurrence or trigger cancer development. In fact, previous studies demonstrated that downregulated miRNAs in bEV of mothers with obesity, such as *miR-630* and *miR-642*, suppress cancers (61, 62). *miR-548j-3p*, upregulated in mothers with obesity, plays a role as a metastasis promoter in breast cancer (63). However, some other miRNAs known as tumor suppressors, including *miR-548-3p* and *miR-652-5p* (64, 65), were slightly upregulated in mothers with obesity. Further studies would be needed to test each miRNA as a breast cancer predictive biomarker for mothers with obesity. More importantly, these miRNAs are eventually transferred to breastfed infants. The impact of these altered cancer-related miRNAs on infants also needs to be considered.

Our study has limitations. First, although it is a pilot study, we haven't been able to include enough samples in the group with obesity. However, we excluded the effect of the overweight population, which may diminish the significance of the result. Second, we analyzed breast milk collected from a wide range of postpartum dates. However, we used mature breast milk that was relatively stable in composition (66), and

we statistically controlled for the potential impact of different postpartum phases. Third, there is a variation in time between milk collection and the analysis of bEV miRNA. While miRNAs are globally stable, individual miRNAs display rapid decay dynamics in some specific situations (67). Although we froze breast milk at −80°C until use and did not repeat the freeze and thaw cycle after RNA extraction until NanoString analysis, there might be a possibility of miRNA degradation that can affect the miRNA profiles.

To conclude, we characterized bEV miRNAs and identified differentially expressed miRNAs of bEVs in mothers with obesity and demonstrated altered miRNAs in bEVs. Altered miRNAs in bEVs of mothers with obesity may influence the neurological and psychological development of breastfed infants. Further longitudinal studies investigating the precise role of altered miRNAs in infants need to be followed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo, GSE212951.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of the University of Iowa (IRB #202005237). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YEC: conceptualization, methodology, resources, writing—original draft preparation, supervision, and funding acquisition. YEC and MC: formal analysis and data curation. YEC, RV, KC, CS, JN, AH, and RS: investigation. YEC, RV, CS, JG, and HL: writing—review and editing. YEC and RV: Visualization and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.976886/full#supplementary-material

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Characteristics and predictors of breast milk iodine in exclusively breastfed infants: Results from a repeated-measures study of iodine metabolism

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Background: The iodine supply of exclusively breastfed infants entirely depends upon breast milk. Changes in breast milk iodine affect infants' iodine nutritional status. This study aimed to comprehensively assess the characteristics and predictors of breast milk iodine concentration (BMIC).

Materials and methods: This 7-day iodine metabolism experiment was conducted in 25 exclusively breastfed mother-infant pairs. The duplicate portion method was used to measure the mother's daily iodine intake from foods and water, and maternal 24-h urine excretion was assessed. We recorded the number of breastfeeds per mother per day and collected breast milk samples before and after each feeding.

Results: The median [quartile (Q)1–Q3 range] of BMIC was 115 (86.7, 172) μg/L. The BMIC before breastfeeding was generally higher than that after breastfeeding. Time-sequential analysis found that morning BMIC was most highly correlated with the prior day's iodine intake. Breast milk samples taken in the afternoon or after midnight are closer to the median level of BMIC throughout the day. The number of breast milk samples needed to estimate the iodine level with 95% CI within precision ranges of ± 20% was 83 for a population, 9 for an individual, and 2 for an individual's single day. Maternal total iodine intake (TII) and urine iodine were significantly associated with BMIC. 24-h urinary iodine excretion (24-h UIE) was found to be the best predictive indicator for the BMIC (β = 0.71, 95% CI: 0.64, 0.79).

Conclusion: BMIC is a constantly changing indicator and trended downward during each breastfeeding. Breast milk samples taken in the afternoon or after midnight are most representative. BMIC was significantly associated with recent iodine intake. Maternal 24-h UIE was the best predictor of BMIC.

KEYWORDS

breast milk iodine, breastfed infants, urine iodine excretion, iodine metabolism study, iodine intake

Introduction

Iodine, an essential micronutrient, is required by humans for the synthesis of thyroid hormones. Although suboptimal iodine intake can lead to a broad spectrum of disorders throughout life (1), adequate iodine intake is most critical in the early stages of development, as the infant's normal neurodevelopment and growth are extremely dependent on the iodine supply. In the first 1,000 days of life, iodine deficiency can cause hypothyroidism and irreversibly impair neurodevelopment (2–4). The World Health Organization (WHO) recommends exclusive breastfeeding during the first 6 months of life, and breast milk is the only source of iodine for exclusively breastfed infants (5). Breast milk iodine thus reflects the iodine nutrition of both mothers and their breastfed infants (5–7).

Several factors such as maternal iodine status and duration of breastfeeding may influence breast milk iodine concentration (BMIC) (5). However, existing studies have only collected spot urinary iodine and breast milk iodine for comparisons, without comprehensively studying the relationship between breast milk iodine and maternal iodine intake (6, 8). Two recent reviews pointed out that the majority of studies on breast milk iodine have collected colostrum or mature milk only once and have had small sample sizes (5, 9). No study protocol involved whole-day breast milk sample collection. Furthermore, although some studies have shown large variations in BMIC (7), there are limited data on intra-individual, inter-individual, and daily variations in BMIC. Currently, studies that comprehensively summarize the characteristics of breast milk iodine are lacking.

The objectives of the study were to comprehensively assess the characteristics of BMIC and to identify parameters associated with breast milk iodine.

Subjects and materials and methods

Study setting and sample

This study was performed in Gaoqing Country of Zibo City in Shandong Province, China. The study was home-based. Inclusion criteria were as follows: (1) lactating women aged 20 to 45 years; (2) no dietary restrictions; (3) no history of thyroid disease; (4) infants of singleton pregnancy; (5) delivery at full term and normal fetal birth weight (2.5–4 kg); (6) infants 0 to 6 months of age; and (7) exclusive breastfeeding. All infants and mothers were in good health. Initially, 32 volunteers were recruited, and after preliminary screening, 25 health mother-child pairs were included in the final analysis. A flowchart is presented in **Figure 1**. The Medical Ethics Committee of Tianjin Medical University approved the research protocols (ethics approval number TMUhMEC2020033). Subjects provided written informed consent before the study. This study was registered at www.clinicaltrials.gov as NCT04492657.

Study design

Iodine intake (diet and water) and 24-h urinary iodine excretion (24-h UIE) of the lactating women were assessed. For infants, we recorded number of feedings per day and retained breast milk samples from each feeding. Researchers who underwent professional training measured infant length and weight on the day of the study. Weight measurements were performed using a special infant weight scale. The instrument was placed on a horizontal table and zeroed before measurement, and the infant was placed on the scale after removing the infant's outer clothing, shoes and hats, and their weight was weighed and recorded. Then the infant was placed supine on a horizontal bed, kept by the investigator on the right side of the infant, holding the infant's knees with the left hand and keeping the legs straight. The other person used a steel ruler to measure the length of the child from the heel to the top of the head. A detailed questionnaire was used to collect information on women's demographics, height, weight, and method of delivery. Blood samples (4–5 mL) were collected for maternal thyroid function measurement. Maternal spot urine and breast milk, and spot infant urine samples were collected. Procedures were explained in detail to participating lactating women. Each mother-child pair was guided by the same professional staff member throughout the study process. Study staff was available to answer subjects' questions and were responsible for the quality control of the sample collection.

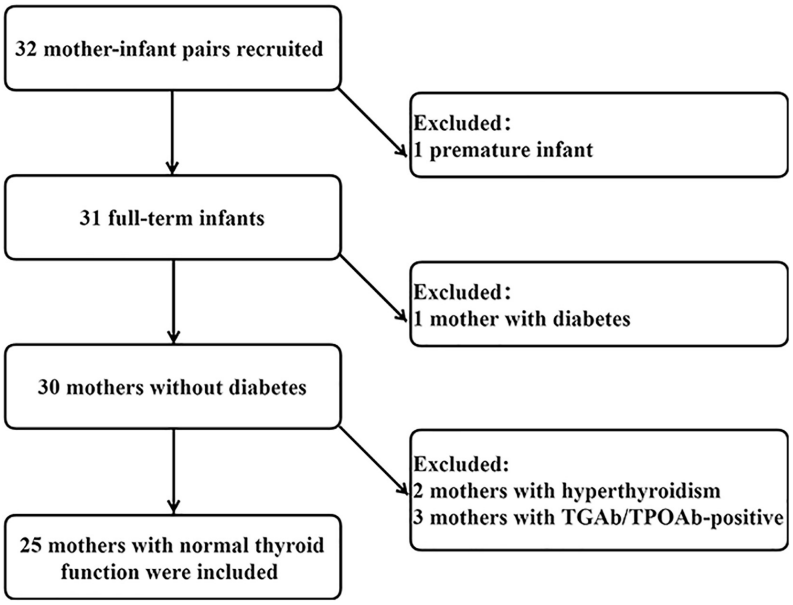

FIGURE 1
Flowchart of the study.

Breast milk sample collection

Subjects were asked to use a breast pump to empty their breasts before starting the study. The times of breast-feeding were not controlled. Breast milk samples were collected before and after each lactation. A breast milk sample collected after 8:00 am each day was the first sample of the day and samples were sequentially collected and recorded thereafter. Eight of 25 subjects recorded the exact time of each breastfeeding. Lactating women were required to retain no less than 2 mL of breast milk samples before and after each breastfeeding. The sample number and collection status were checked by study staff daily. Samples were stored at −20°C and tested within 2 weeks after collection.

Dietary sample collection

The duplicate portion method was adopted to collect dietary specimens for seven consecutive days. Diet data were collected at the same time as the 24-h urine samples, all for 24 h. During the study, all the subjects were provided with a kitchen scale and required to accurately weigh all foods they ate. Duplicates of foods and beverages consumed during the day were systematically collected to determine and calculate daily dietary iodine intake. In order to further ensure the accuracy of dietary specimen retention, each subject was required to fill out a daily dietary record. Investigators collected and classified food samples and checked them against the dietary record forms each morning. Food samples were stored in vacuum sealed bags and transported to the laboratory. In the laboratory, food samples were weighed, mashed and homogenized. The homogenized 5–10 g samples were stored in a freezer at −20°C until analysis.

Drinking water sample collection

The drinking water intake assessment included only pure water or tap water for drinking. Homemade hot beverages such as coffee, milk, tea, etc., are retained and measured separately for their iodine content and are classified as food iodine. Each participant was provided with a calibrated drinking glass. The amount of water consumed within 24 h was recorded. Five ml drinking water samples were retained, stored at 4°C and tested within 2 weeks. The daily iodine intake from drinking water was calculated.

Urine sample collection

Every participant was provided with a 2.5-liter iodine-free plastic drum and a beaker with a handle. 24-h urine specimens were collected from 8:00 am to 8:00 am the next day. All urine was collected in plastic buckets. Investigators measured the volume of the urine samples. After mixing, 5 ml urine aliquots were stored at 4°C, and tested within 2 weeks. The 24-h urinary iodine excretion was obtained by multiplying the 24-h urinary iodine concentration by the 24-h urine excretion volume. Spot urine samples from mothers and infants were collected once using urine cups and urine collection bags one day before the start of the iodine metabolism study.

Laboratory analyses

Breast milk iodine concentration (BMIC) was analyzed by inductively coupled plasma mass spectrometry (ICP-MS; iCAP Q, Thermo Fisher Scientific). ICP-MS was operated with argon (> 99.999%, high purity) in kinetic energy discrimination for the determination of elemental iodine. The flow rate of argon gas was set as follows: auxiliary: 0.8 L/min; nebulizer: 1.11 L/min; cooling gas flow rate: 14.00 L/min. The isotopes used for the measurements were 127I and 130Te. The standard reference material for whole milk powder (NIST 1549a; National Institute of Standards and Technology) was used for quality control. The intra- and inter- assay CVs were 1.27 and 2.95%, respectively. ICP-MS was also used for determination of water iodine concentration (WIC), food iodine, and urine iodine. Water samples were diluted 11-fold with 7 mmol/L hydrous ammonium and 100 μg/L Te. The inter-assay and intra-assay CV for drinking water measurement were 1.8–3.0 and 0.7–2.3%, respectively. Food samples were digested with 25% tetramethylammonium hydroxide at 90°C for 5 h. For food iodine samples, the standardized reference materials were purchased from National Institute of Standards and Technology (SRM 1548a for typical diet). The mean iodine concentration assessed by the method was 0.733 ± 0.035 mg/kg, compared to the reference value of 0.759 ± 0.103 mg/kg. The inter-assay and intra-assay CV were 1.5–2.6 and 2.8–4.0%, respectively. The analytical recovery from food was 98–110%. Urine samples were diluted 21-fold with 0.25% TMAH, 0.02% Triton X-100, and 100 μg/L Te before analysis using ICP-MS. The total inter-assay and intra-assay CV% for urinary iodine concentration (UIC) measurements were 1.4–3.2 and 0.6–1.8%, respectively. Thyroid function parameters, including serum free triiodothyronine (FT3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH), were determined using the ADVIA Centaur CP Immunoassay System (Siemens). The lowest detection limits of TSH, FT4, and FT3 were 0.008 mIU/L, 1.3 and 0.3 pmol/L, respectively. Quality-control processes were performed according to the manufacturer's instructions before, during, and after the testing. The intra-assay CVs for serum TSH, FT4 and FT3 were 2.1–4.9, 1.7–4.2, 2.4–3.1%, respectively. The inter-assay CVs for serum TSH, FT4 and FT3 were 1.5–4.4, 1.4–3.1, and 2.8–4.1%, respectively.

Statistical analyses

Total iodine intake (TII) is the sum of water iodine and dietary iodine. We summarize normally distributed data as mean ± SD and non-normally distributed data as the median [quartile (Q)1–Q3 range]. Wilcoxon matched-pairs signed-ranks test was used to assess differences in BMIC before and after lactation. Spearman rank correlation was used to examine the correlations between BMIC and TII, UIC, and UIE. The estimated sample sizes with specified accuracy were calculated by the formula: $N = (Z \times CV/D)^2$ (10). The coefficient of variation (CV) was the standard deviation divided by the mean, as a proportion. The precision range (D) used to estimate the required sample size at precision levels varied from ± 1% to ± 50% (10, 11). The confidence interval (CI) value was set to 95%, the corresponding Z value was 1.96. A sensitivity analysis was performed to determine changing trends in BMIC by infant age. Physical development and BMIC consumed by infants of different weeks of age were compared using non-parametric Kruskal-Wallis tests. Linear mixed-effects models with per-subject random intercept were performed using PROC MIXED in SAS software for the longitudinal analyses of the factors associated with breast milk iodine. The following variables entered the final model separately as fixed effects: lactating women's BMI, infants' weeks of age, height and weight, TII, 24-h UIE, 24-h UIC, and the prior day's TII, 24-h UIE, and 24-h UIC. We subsequently adjusted for the mother's age, parity and the infant's sex in the analysis of the baseline independent variables. For repeated independent variables, we adjusted for the following potential confounders: maternal age, parity, and BMI and the infants' sex, gestational age at birth, birth height, birth weight, weeks of age, and current height and weight. Missing data were not included in the analysis. Analyses were performed using SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Baseline data

An iodine metabolism study was performed for seven consecutive days. One of the subjects underwent the experiment for 5 days. None of the subjects consumed sea fish, kelp or iodized salt during the experiment. Baseline characteristics are shown in **Table 1**. Lactating women had a mean age of 30.6 ± 6.3 years and all had normal thyroid function. The median (Q1, Q3) of TII, 24-h UIC, and 24-h UIE were 155 (90.0, 302) μg/d, 89.5 (55.7, 134) μg/L, and 106 (65.4, 162) μg/d, respectively. The infants, including 13 boys and 12 girls, aged 15.1 ± 4.9 weeks, were born *via* vaginal delivery and were exclusively breastfed. The median (Q1, Q3) of BMIC was 115 (86.7, 172) μg/L. The mothers' median spot urinary iodine concentration was significantly lower than that of the infants. The infants' length, weight and head circumference increased significantly with age (**Supplementary Table 1**). A gradual decline in median BMIC was observed from 5 to 26 weeks postpartum (P for trend = 0.04).

TABLE 1 Characteristics of lactating women and their infants.

Variables	
mother-infant pairs (n)	25
lactating women	
Age, years	30.6 ± 6.3
BMI, kg/m^2	24.9 ± 3.0
Exclusive breastfeeding, n (%)	25 (100)
Number of pregnancies	
1, n (%)	6 (24.0)
2, n (%)	11 (44.0)
3, n (%)	7 (28.0)
4, n (%)	1 (4.0)
Parity	
1, n (%)	8 (32.0)
2, n (%)	14 (56.0)
3, n (%)	3 (12.0)
FT3, pmol/L	4.7 ± 0.4
FT4, pmol/L	13.2 ± 1.7
TSH, mIU/L	1.6 ± 0.8
WI, mL/d	1357 (900, 2053)
TII, μg/d	155 (90.0, 302)
Spot UIC, μg/L	93.0 (50.1, 169)
24-h UIC, μg/L	89.5 (55.7, 134)
24-h UIE, μg/d	106 (65.4, 162)
Infants	
Gender	
Boys, n (%)	13 (52.0)
Girls, n (%)	12 (48.0)
Weeks of age	15.1 ± 4.9
Birth length, cm	50.0 ± 0.9
Birth weight, kg	3.5 ± 0.5
Current length, cm	63.8 ± 4.0
Current weight, kg	7.3 ± 1.1
Spot UIC, μg/L	195 (138, 301)
Mean BMIC, μg/L	118 (90.1, 181)
Median BMIC, μg/L	115 (86.7, 172)

Data are presented as ratios or means ± SDs or median (Q1, Q3). BMI, body mass index; BMIC, breast milk iodine concentration; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone; TII, total iodine intake; UIC, urinary iodine concentration; UIE, urinary iodine excretion; WI, water intake.

Correlations and differences in breast milk iodine concentration before and after lactation

A total of 1,471 pairs of breast milk samples before and after breastfeeding were collected (Table 2). The BMIC concentrations before and after each feeding were closely correlated. A Wilcoxon matched-pairs signed-ranks test found that the BMIC before lactation [120 (85.3, 190) μg/L] was generally significantly higher than that after lactation [112 (81.6, 174) μg/L] ($P < 0.001$). However, the difference between before- and after-feeding BMIC was inconsistent in different feeding durations. The difference in BMIC between before-feeding and after-feeding was sometimes significant and sometimes not significant during a single feeding.

Correlations between breast milk iodine concentration and maternal iodine intake and urine iodine

Table 3 shows the correlations between BMIC at each time point and total maternal iodine intake, UIE and UIC. BMIC was positively correlated with TII, UIE, and UIC on the same day or in the previous day. The BMIC gradually became more correlated with the TII and urine iodine over time on the same day. However, the correlation with the previous day's iodine intake and urine iodine gradually became smaller as each day progressed. The correlation between BMIC and 24-h UIE was greatest, suggesting that the excretion of breast milk iodine is similar to that of urine iodine. In addition, the correlation between BMIC and TII the prior day was higher than that on the same day when for the first three feedings of each day, which indicates that BMIC in the morning is strongly influenced by the prior day's TII.

Optimal sampling time of breast milk iodine

The median BMIC is often used to represent a day's milk iodine level. Table 4 presents the correlation coefficients between the BMIC at each time point and the median or mean BMIC in a day. Estimating the optimal collection time based on breastfeeding times is only a rough estimate. We can see that the median iodine concentration of milk samples collected at the fourth and fifth feeds and after the 12th feed of the day are closest to the median BMIC ($r = 0.96$). Similarly, we found that the BMIC for samples collected at the fourth and fifth feedings, and after the 10th feeding was closest to the median BMIC of the day in the eight lactating mothers who accurately recorded the sampling time. These two time points are roughly in the afternoon or after midnight.

Variation in breast milk iodine

We evaluated the BMIC variation from multiple perspectives (Table 5). The mean, median, and lowest and highest CV for BMIC in an individual were 34.3, 31.4, 22.2, and 57.1%, respectively. These values were 19.1, 15.5, 1.15, and 78.6%, respectively, in an individual on a single day. The population median (Q1, Q3) of CV in BMIC was 93.2% (91.1, 102%). The median variation in BMIC consumed by infants was

TABLE 2 Difference and correlation of breast milk iodine concentration (BMIC) before and after lactation.

Code	Pairs (n)	Before-BMIC (μg/L)	After-BMIC (μg/L)	*CC*	$p^{\#}$
IB01	58	50.0 (43.6, 58.8)	50.6 (44.1, 59.2)	0.95*	0.17
IB02	84	128 (103, 153)	126 (109, 152)	0.74*	0.32
IB03	40	95.6 (79.0, 114)	93.9 (77.7, 113)	0.99*	0.002
IB04	62	111 (92.5, 152)	97.9 (81.7, 143)	0.89*	<0.001
IB05	76	126 (107, 145)	109 (94.8,134)	0.93*	<0.001
IB06	82	94.7 (80.7, 112)	94.0 (79.6, 108)	0.93*	0.37
IB07	73	56.7 (45.7, 69.8)	54.4 (44.9, 69.1)	0.90*	0.005
IB08	67	105 (92.4, 124)	97.1 (86.4, 111)	0.87*	<0.001
IB09	35	591 (397, 698)	496 (393, 603)	0.89*	0.03
IB10	51	179 (140, 243)	162 (123, 211)	0.88*	<0.001
IB11	101	297 (212, 365)	327 (176, 405)	0.80*	0.10
IB12	59	77.4 (56.1, 118)	74.8 (54.7, 112)	0.95*	0.001
IB13	28	184 (160, 216)	161 (148, 203)	0.87*	<0.001
IB14	48	116 (110, 133)	109 (102, 117)	0.42*	0.001
IB15	61	74.1 (62.8, 84.1)	71.1 (58.0, 82.3)	0.88*	0.003
IB16	37	156 (112, 177)	139 (102, 186)	0.86*	0.06
IB17	61	69.6 (61.6, 83.4)	70.5 (59.1, 82.0)	0.86*	0.96
IB18	35	94.6 (84.9, 107)	94.3 (74.0, 106)	0.83*	0.29
IB19	65	121 (101, 219)	154 (102, 238)	0.51*	0.10
IB20	78	701 (572, 797)	743 (608, 832)	0.70*	<0.001
IB21	56	126 (111, 134)	125 (107, 140)	0.66*	0.74
IB22	58	153 (128, 190)	118 (106, 144)	0.83*	<0.001
IB23	66	94.9 (60.1, 149)	90.5 (51.7, 135)	0.90*	0.002
IB24	64	214 (185, 243)	182 (152, 209)	0.69*	<0.001
IB25	26	127 (117, 169)	128 (111, 155)	0.20	0.42
Total	1471	120 (85.3, 190)	112 (81.6, 174)	0.94*	<0.001

Data are presented as medians (25th, 75th percentiles). After-BMIC, breast milk iodine concentration after a feeding; Before-BMIC, breast milk iodine concentration before a feeding; CC, correlation coefficient. CC was calculated by the Spearman Rank correlation test.
#Made by the Wilcoxon matched-pairs signed-ranks test.
*Indicates statistical significance ($P < 0.05$).

lower than maternal 24-h UIC, 24-h UIE, and TII. However, the population median variation of 24-h UIC and BMIC is similar.

Eight of twenty-six subjects recorded the time of each breastfeeding. The change in BMIC after 8:00 am on the first day of the experiment is shown in Figure 2. We can see that the BMIC levels in most lactating mothers fluctuate relatively smoothly. One lactating mother had a particularly high level of BMIC. In addition, we estimated each breastfeeding time for the remaining 18 lactating mothers based on the number of breastfeeds per day. The fluctuations in BMIC in all subjects are shown in Supplementary Figure 1.

Sample sizes for iodine nutrition monitoring

Table 6 lists the number of samples necessary to estimate breast milk iodine status in a population, an individual, and an individual day with 95% confidence. The sample size varies with the different CVs and precision ranges. Specifically, in a study of 334 participants providing one breast milk sample, the precision range will be about ± 10%. This means that, if the mean BMIC in a survey was 150 μg/L, the true mean BMIC in that population would be between 135 and 165 μg/L. According to the median CV, obtaining a precision range of ± 10% in an individual would require thirty-eight breast milk samples. Furthermore, if we want to estimate the iodine content of a person's breast milk on a given day, collecting two samples from an individual in a day would achieve a ± 20% precision range.

Parameters associated with breast milk iodine

A mixed-effects regression analysis was used to analyze the factors associated with BMIC (Table 7). Infant week of age, height, and weight were not related to BMIC. Maternal TII and urine iodine were significantly correlated with BMIC. In particular, the maternal 24-h UIE was found to be the best predictor for the level of BMIC (β = 0.71, 95% CI: 0.64, 0.79). In addition, the prior day's TII (β = 0.06, 95% CI: 0.03, 0.09),

TABLE 3 Correlations between breast milk iodine concentration (BMIC) and total iodine intake (TII), urinary iodine excretion (UIE) and urinary iodine concentration (UIC) on the same day and the prior day.

BMIC collection times	Same day							Prior day						
	N	TII		24-h UIC		24-h UIE		*N*	TII		24-h UIC		24-h UIE	
		CC	*P*	CC	*P*	CC	*P*		CC	*p*	CC	*P*	CC	*P*
1	170	0.50	<0.001	0.55	<0.001	0.65	<0.001	146	0.62	<0.001	0.63	<0.001	0.72	<0.001
2	168	0.49	<0.001	0.55	<0.001	0.66	<0.001	143	0.57	<0.001	0.62	<0.001	0.70	<0.001
3	171	0.56	<0.001	0.58	<0.001	0.70	<0.001	146	0.59	<0.001	0.61	<0.001	0.71	<0.001
4	170	0.60	<0.001	0.63	<0.001	0.70	<0.001	145	0.55	<0.001	0.60	<0.001	0.67	<0.001
5	170	0.64	<0.001	0.63	<0.001	0.72	<0.001	145	0.54	<0.001	0.53	<0.001	0.62	<0.001
6	156	0.61	<0.001	0.67	<0.001	0.72	<0.001	133	0.55	<0.001	0.47	<0.001	0.53	<0.001
7	144	0.56	<0.001	0.63	<0.001	0.73	<0.001	123	0.55	<0.001	0.44	<0.001	0.54	<0.001
8	129	0.51	<0.001	0.62	<0.001	0.72	<0.001	110	0.50	<0.001	0.44	<0.001	0.52	<0.001
9	100	0.52	<0.001	0.56	<0.001	0.75	<0.001	83	0.49	<0.001	0.34	0.002	0.50	<0.001
10	68	0.71	<0.001	0.59	<0.001	0.84	<0.001	57	0.56	<0.001	0.25	0.06	0.57	<0.001
11	49	0.73	<0.001	0.48	0.001	0.85	<0.001	42	0.56	0.0001	0.30	0.05	0.64	<0.001
N ≥ 12	69	0.75	<0.001	0.56	<0.001	0.91	<0.001	55	0.75	<0.001	0.33	0.01	0.69	<0.001
Total[a]	1564	0.58	<0.001	0.59	<0.001	0.72	<0.001	1328	0.57	<0.001	0.51	<0.001	0.63	<0.001
Total[b]	173	0.60	<0.001	0.65	<0.001	0.76	<0.001	148	0.57	<0.001	0.55	<0.001	0.64	<0.001
Total[c]	173	0.62	<0.001	0.64	<0.001	0.74	<0.001	148	0.57	<0.001	0.55	<0.001	0.64	<0.001

[a]Based on all breast milk samples. BMIC is the average breast iodine concentration before and after lactation. [b]BMIC is the mean iodine concentration of the breast milk samples for a given a day. [c]BMIC is the median iodine concentration of the breast milk samples for a given a day. BMIC, breast milk iodine concentration; CC, Correlation Coefficient; TII, total iodine intake; 24-h UIC, 24-h urinary iodine concentration; 24-h UIE, 24-h urinary iodine excretion.

TABLE 4 Estimation of the optimal sampling time of breast milk iodine.

BMIC collection times	Twenty-five subjects[a]				BMIC collection times	Eight subjects[b]				
	N	Mean BMIC in a day		Median BMIC in a day			*N*	Mean BMIC in a day		Median BMIC in a day	
		CC	*P*	CC	*P*			CC	*P*	CC	*P*
1	170	0.87	<0.001	0.88	<0.001	1	56	0.87	<0.001	0.88	<0.001
2	168	0.88	<0.001	0.88	<0.001	2	55	0.88	<0.001	0.87	<0.001
3	171	0.92	<0.001	0.94	<0.001	3	56	0.94	<0.001	0.94	<0.001
4	170	0.94	<0.001	0.96	<0.001	4	56	0.94	<0.001	0.96	<0.001
5	170	0.94	<0.001	0.96	<0.001	5	54	0.92	<0.001	0.95	<0.001
6	156	0.95	<0.001	0.93	<0.001	6	54	0.91	<0.001	0.89	<0.001
7	144	0.94	<0.001	0.89	<0.001	7	52	0.92	<0.001	0.88	<0.001
8	129	0.93	<0.001	0.88	<0.001	8	49	0.87	<0.001	0.82	<0.001
9	100	0.94	<0.001	0.91	<0.001	9	39	0.92	<0.001	0.89	<0.001
10	68	0.95	<0.001	0.95	<0.001	N ≥ 10	47	0.94	<0.001	0.95	<0.001
11	49	0.91	<0.001	0.92	<0.001						
N ≥ 12	69	0.96	<0.001	0.96	<0.001						

[a]Twenty-five subjects recorded the number of feedings and collected breast milk before and after each feeding. [b]Eight of twenty-five subjects recorded the time of each breastfeeding and collected breast milk before and after each feeding. The BMIC for each lactation is the average BMIC before and after lactation. The mean BMIC in a day is the mean iodine concentration of the breast milk samples for a given day. The median BMIC in a day is the median iodine concentration of the breast milk samples for a given day.

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TABLE 5 Variation in iodine nutrition indicators.

CV	BMIC (μg/L)			24-h UIC (μg/L)		24-h UIE (μg/d)		TII (μg/d)	
	A population	An individual	An individual a day	A population	An individual	A population	An individual	A population	An individual
Min	78.0%	22.2%	1.15%	60.7%	11.0%	54.2%	9.2%	106%	12.5%
Mean	99.4%	34.3%	19.1%	90.9%	38.5%	74.0%	35.3%	118%	54.6%
Q1	91.1%	27.3%	11.4%	78.0%	27.8%	66.3%	28.9%	108%	28.5%
Median	93.2%	31.4%	15.5%	94.2%	41.3%	77.1%	33.1%	114%	52.1%
Q3	102%	37.2%	22.9%	99.9%	46.8%	80.3%	43.9%	128%	76.2%
Max	139%	57.1%	78.6%	125%	73.1%	93.4%	60.6%	133%	107%

BMIC, breast milk iodine concentration; CV, coefficient of variation; 24-h UIC, 24-h urinary iodine concentration; 24-h UIE, 24-h urinary iodine excretion.

24-h UIC (β = 0.20, 95% CI: 0.10, 0.30) and 24-h UIE (β = 0.32, 95% CI: 0.23, 0.41) were related to the day's BMIC.

Discussion

Compared with other age groups, neonates and infants are susceptible to iodine deficiency as they have lower intrathyroidal iodine storage and the highest iodine requirements relative to body weight (9–12). Breast milk is the only iodine source for exclusively breastfed infants. Appropriate breast milk iodine levels are required for infants' normal physical and neurologic growth and maturation (13). The mother's body has physiological mechanisms for iodine regulation during lactation. Iodide transport and uptake by the mammary gland are mediated by the sodium-iodide symporter (NIS), the expression of which is increased in the lactating breast (14, 15). However, the breast's ability to regulate iodine concentrations seems to be limited (16), as breast milk iodine concentrations vary widely (9). BMIC ranges of 5.4–2,529 μg/L have been reported, even in populations considered iodine sufficient (5, 7, 17–20).

Our study site has been regarded as iodine sufficient based on local drinking water iodine and urine iodine monitoring data. The median BMIC was 115 μg/L in our study. In general, the volume of breast milk ingested by infants aged 0–6 months is about 500–1,000 ml/day, similar to what we observed. The Chinese iodine Adequate Intake (AI) for infants aged 0–6 months was based upon a median BMIC of 112 μg/L multiplied by an average milk excretion of 0.75 L/day to approximately equal 85 μg/day (21). The infant iodine AI defined by the U.S. Institute of Medicine (IOM) was 110 μg/day and the recommended infant iodine intake defined by the WHO was 90 μg/day. If the optimal BMIC is inferred from the recommended iodine intake and a breast milk volume of 500–1,000 ml/day, the normal BMIC range is 85–220 μ g/L.

A trend toward a moderate decrease in BMIC over the course of lactation has been found in longitudinal studies in iodine-deficient areas (22, 23), iodine-sufficient areas (8, 24), and iodine-excessive areas (25). We also observed a gradual decrease in median BMIC with increasing infant age and body mass from 5 to 26 weeks postpartum. In addition, we found that BMIC before each feeding was generally higher than that after ($P < 0.001$), indicating there is a dynamic change in BMIC during each breastfeeding. When Dold et al. required subjects to provide three consecutive breast milk samples from one feeding session, they similarly found that iodine content was significantly lower in the post-feeding samples compared to the pre-and mid-feeding breast milk samples (26). A previous study pointed out that the lower BMIC in the post-feeding period may be the result of physiological changes in the composition of breast milk during feeding. There is a decrease in the proportion

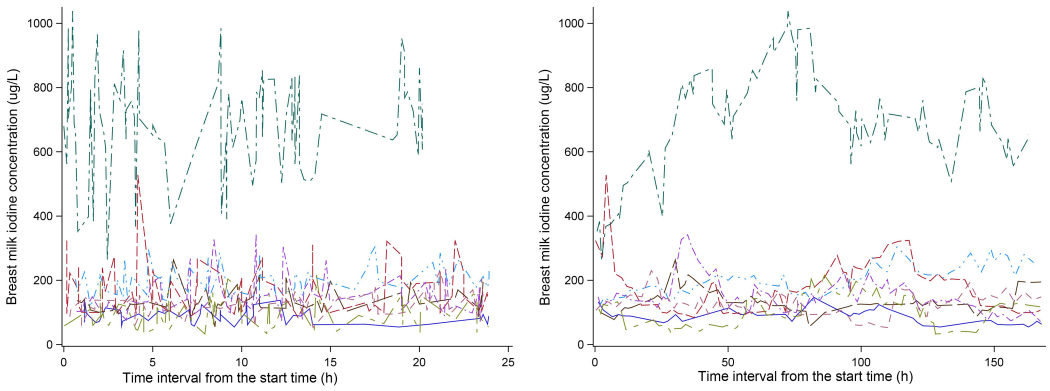

FIGURE 2
The changing trend of breast milk iodine concentration in eight lactating women.

TABLE 6 Number of samples needed to be 95% confident of being within a specified range for estimating the breast milk iodine status.

Precision ranges	A population			An individual			An individual a day		
	Median CV	Lowest CV	Highest CV	Median CV	Lowest CV	Highest CV	Median CV	Lowest CV	Highest CV
± 1%	33369	23372	74224	3788	1893	12525	923	5	23733
±2%	8342	5843	18556	947	473	3131	231	1	5933
± 5%	1335	935	2969	152	76	501	37	1	949
±10%	334	234	742	38	19	125	9	1	237
± 20%	83	58	186	9	5	31	2	1	59
±30%	37	26	82	4	2	14	1	1	26
± 40%	21	15	46	2	1	8	1	1	15
±50%	13	9	30	2	1	5	1	1	9

Calculated from $N = (Z \times CV/D)^2$, where $Z = 1.96$ for 95% CI and D is the precision range. CV, coefficient of variation.

of the iodine-containing aqueous phase due to an increase in the fat content of breast milk at the end of feeding (27). Although some studies have found no differences in BMIC before and after lactation (7, 28, 29), paired tests in our study demonstrated that BMIC sometimes does differ significantly before and after lactation. We speculate that the differences in milk iodine concentration before and after a feeding may be related to the duration of a single feeding.

Moreover, we described diurnal changes in BMIC. The correlations between BMIC collected after 8:00 am and the day's TII, 24-h UIE, and 24-h UIC gradually increased over the course of each day. In contrast, the correlation with the prior day's TII and urinary iodine level progressively decreased. BMIC was more highly correlated with 24-h UIE than with TII and 24-h UIC, which is consistent with the results of a prior Danish study (30). Although the changing patterns of BMIC are similar to those of urinary iodine, BMIC level is primarily determined by the maternal dietary iodine intake (16, 31, 32). BMIC responds quickly to recent changes of dietary iodine intake (within hours), either from supplements or food (30, 33). The ingested iodine is rapidly excreted in breast milk, and the BMIC peaks within 6 h after consumption (16, 33).

Substantial BMIC day-to-day intra-individual variability (1.15–78.6%) was observed in our study. Kirk et al. described considerable diurnal variation in BMIC, but no specific variability values were given (34). A recent review found no consistent trends in BMIC by the time of day (9), although our study demonstrates irregular diurnal fluctuations in BMIC. Overall, we found the population variability of BMIC was significantly higher than the inter-individual variability. According to prior studies, BMIC is widely variable across populations, which is likely influenced by habitual and recent maternal iodine intake and status (9). In our study, the variability in TII was the largest, which is consistent with the findings of Chen et al. (35). The variability of BMIC and urinary iodine within the population and for each individual were roughly similar, which confirms that the excretion rhythms of milk and urinary iodine are similar. The number of breast milk samples needed to estimate the iodine level in a population in a day with 95% confidence within a precision range of ± 20%

TABLE 7 Linear mixed-effects models of predictors of breast milk iodine concentration (BMIC).

Variables	Unadjusted			Adjusted		
	β	95% CI	*p*	β	95% CI	*p*
Lactating women's BMI[a]	15.5	−3.92, 35.0	0.12	17.2	−4.07, 38.5	0.11
Infant's weeks of age[a]	−3.79	−16.4, 8.80	0.56	−3.09	−17.2, 11.0	0.67
Infant's height[a]	−5.64	−20.8, 9.48	0.47	−5.43	−22.8, 12.0	0.54
Infant's weight[a]	−2.92	−36.6, 30.7	0.86	−1.37	−40.7, 38.0	0.95
TII[b]	0.09	0.06, 0.12	<0.001	0.08	0.05, 0.11	<0.001
24-h UIC[b]	0.54	0.46, 0.62	<0.001	0.54	0.46, 0.62	<0.001
24-h UIE[b]	0.72	0.65, 0.80	<0.001	0.71	0.64, 0.79	<0.001
the prior day's TII[b]	0.07	0.03, 0.10	<0.001	0.06	0.03, 0.09	<0.001
the prior day's 24-h UIC[b]	0.20	0.11, 0.30	<0.001	0.20	0.10, 0.30	<0.001
the prior day's 24-h UIE[b]	0.33	0.25, 0.42	<0.001	0.32	0.23, 0.41	<0.001

[a]Adjusted for the mother's age, parity, and the infant's sex.
[b]Adjusted for the mother's age, parity, BMI and the infant's sex, gestational age at birth, birth height, birth weight, weeks of age, current height and weight.

was about two. We recommend collecting samples after lunch or after midnight. To estimate an individual's iodine level with 95% confidence within a precision range of ± 20%, the breast milk sample size required (9 samples) is lower than that of spot urine (14 samples) (36). Although urinary iodine concentration is commonly used to assess population iodine nutritional status, BMIC may be the preferred index for infants (6, 37).

In this study, maternal 24-h UIE was found to be the best predictor for the level of BMIC. The positive correlation between BMIC and 24-h UIE was stronger than the correlation with dietary iodine intake. Accordingly, we speculate that the iodine excretion function of the mammary gland is similar to that of the kidney under specific conditions. This excretion mechanism is relatively stable in a well-nourished population. Several studies have found the positive correlation between BMIC and creatinine-adjusted UIC is stronger in iodine-sufficient populations (9, 30). BMIC has been shown to be independent of maternal fluid intake (30, 38, 39), while the UIC is affected by it.

Strengths and limitations

This is the first study to comprehensively summarize characteristics and predictors of BMIC based on a repeated measures study of iodine metabolism. We accurately measured dietary iodine intake and 24-h urinary iodine excretion. Each nursing mother collected all milk samples before and after each feeding for each day, and eight of them recorded the time of each feeding. The differences in BMIC before and after each feeding as well as the diurnal variation trends and individual and population variability of BMIC were determined. Additionally, the representative optimal sampling time was proposed, and the significant related factors of breast milk iodine level were identified.

Our study has some limitations. First, sampling time was not recorded by all participants. Estimates of sampling time in this study were based on samples collected from 8 of the 25 subjects. Although we roughly estimated the sampling time according to the daily number of samples, the optimal sampling time for BMIC needs to be verified by future studies. Second, we did not record the time between the previous meal of the mother and the milk sampling. Third, the sample size of this study was relatively small. In estimating of the appropriate ranges for BMIC, we only considered that the infant was healthy and the iodine intake was appropriate, and we did not explore reasons for high or low milk iodine concentrations. Large-scale population iodine nutrition epidemiological surveys, including in populations with thyroid diseases, will be needed to clarify the reasons for the large variations in BMIC.

Conclusion

The correlation between BMIC and daily iodine intake and urinary iodine increased gradually over the course of each day. Breast milk samples collected in the afternoon or after midnight are closest to the median breast milk iodine level throughout the day. BMIC was significantly associated with recent iodine intake, with an excretion pattern similar to 24-h UIE.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**,

further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Tianjin Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

WG: visualization, investigation, writing—original draft, funding acquisition, and formal analysis. WW and SL: investigation and writing—original draft. EP and ZR: writing—original draft. MG: investigation and data curation. ZP, NZ, and KZ: investigation. YY: conceptualization, supervision, and investigation. WZ: funding acquisition and project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1017744/full#supplementary-material

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Vitamin E concentration in breast milk in different periods of lactation: Meta-analysis

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Objective: This study systematized information about vitamin E concentration in healthy breast milk during different stages of lactation in order to support the strategies of protecting postpartum women and infants.

Methods: Studies published before April 30th, 2021, which detected vitamin E concentration in breast milk of healthy women by High Performance Liquid Chromatography (HPLC) or Ultra High Performance Liquid Chromatographic (UHPLC), were evaluated. The databases of CNKI (Chinese), WanFang Data (Chinese), VIP (Chinese), PubMed, Cochrane Library, Web of Science and Embase were searched. The random effect models were used to conduct meta-analysis by the statistical software package Stata 14.0.

Results: In all 4,791 searched publications, 53 with full text were selected, which included 46 descriptive studies, 1 case-control study, 1 non-randomized controlled trial, and 5 randomized controlled trials. The pooled mean of vitamin E concentration was 10.57 mg α-TE/L (95%CI 8.94–12.20) in colostrum, 4.03 mg α-TE/L (95%CI 3.29–4.77) in transitional milk and 3.29 mg α-TE/L (95%CI 2.95–3.64) in mature milk. Subgroup analysis showed that vitamin E concentration of colostrum in Asian countries was lower than that in Western countries in colostrum and transitional milk.

Conclusions: Vitamin E concentration in breast milk decreased during lactation until the mature milk was produced. The vitamin E concentration of colostrum in Asian countries was evidently lower than that in Western countries. The vitamin E concentration in mature milk is similar in different regions. The concentration of vitamin E in breast milk started to be stable from about 2 to 3 weeks postpartum until 4 or 6 months postpartum, but it needs additional evidence to support.

KEYWORDS

vitamin E, alpha-tocopherol, breast milk, lactation, meta-analysis

Introduction

Breast milk is important for infant growth and development, which is the most convenient and accessible source of nutrition for infants in the first 6 months of life. The exclusive breastfeeding is recommended for the first 6 months and then continued breastfeeding alongside appropriate complementary foods from thereafter to 24 months. Consequently, studying the composition of breast milk is of crucial importance (1, 2).

Vitamin E, also known as tocopherol, functions as a potent antioxidant, which protects cells from oxidative damage and maintains normal immunity. It is closely related to the development of respiratory, immune and cognitive systems in infants (3). It comprises a group of compounds possessing tocopherol and tocotrienol and their derivatives. Vitamin E includes four tocopherols and four tocotrienols designated as α-, β-, γ-, and δ-. α-tocopherol, which is preferentially recognized by the α-tocopherol transfer protein (TTP) in the human body, is the compound playing the highest vitamin E activity (4, 5).

The nutritional supplement of vitamin E to the fetus through the placenta is limited during pregnancy. Therefore, postpartum breastfeeding has become a significant source for infants to obtain vitamin E. This way of vitamin E supplementation could help infants defend oxygen toxicity in the extrauterine environment and protect the lipoproteins and polyunsaturated fatty acids present in the cellular membranes against peroxidation (6). As described above, the content of vitamin E in breast milk is extremely vital for babies.

This review systematically searched and analyzed three databases for Chinese language articles, four databases for English language articles to obtain more comprehensive information. The aim of the present meta-analysis was to systematize information about vitamin E concentration in healthy breast milk during different periods of lactation, which might be useful to establish support strategies to protect postpartum women and infants.

Materials and methods

This meta-analysis was conducted according to the norms of Meta-Analysis of Observational Studies in Epidemiology (MOOSE) (7), with the following questions: What are the vitamin E concentrations in different periods of lactation of healthy breast milk? Do the vitamin E concentrations in breast milk of normal mothers vary in different regions?

Search strategy

Studies published before May 2021 were searched by three independent reviewers in databases of CNKI (Chinese), WanFang Data (Chinese), VIP (Chinese), PubMed, Cochrane Library, Web of Science and Embase. The following key words were used (Table 1). Authors were contacted when full-text of articles were not available.

Eligibility criteria

We adopted as inclusion criteria the studies that:

- language was Chinese or English;
- involved lactating mothers aged from 18 to 45 years old in addition to infants aged from 0 to 48 months;
- either mothers or infants were medically certified as healthy;
- involved intervention studies and observational studies (cross-sectional study, case-control study, cohort study). Lactating mothers in control group in randomized controlled trials, who did not intake special dietary or participate in dietary supplementation, were included in this meta-analysis;
- the concentration of vitamin E in breast milk was detected by High Performance Liquid Chromatography (HPLC) or Ultra High Performance Liquid Chromatographic (UHPLC).

The studies were excluded that:

- lactating mothers were active smokers, or with chronic conditions (such as gestational diabetes or mastitis), or undergoing pharmacotherapy;
- lactating mothers received interventions from special diets or dietary supplements;
- lactation stages were not described distinctly;
- the main outcomes did not have values;
- included conference papers, reviews, ecological studies, case reports, editorials, letters, commentary, short surveys, and notes.

Study selection and data extraction

The workflow is presented in Table 1. First, duplicate studies were removed manually or by using Endnote. Next, titles and abstracts screening were performed in order to exclude the irrelevant studies. Full-text articles which needed further investigation were assessed by eligibility criteria.

Two researchers screened information and extracted the data independently, and disagreements were resolved by consensus. When a consensus could not be reached, the third reviewer was consulted. The following information was extracted from the final included articles, which included the first author, year of publication, country, lactation stage, sample

TABLE 1 Literature search.

Databases	Key words
CNKI/WanFang Data/VIP	The research strategy of Chinese databases adopted included different combinations of the following terms in Chinese: "Vitamin E", "tocopherol", "breast milk", "mother milk", "breastfeed".
PubMed/Cochrane Library	('Vitamin E'*[Title/Abstract] OR tocopherol[Title/Abstract]) AND ('breast milk'[Title/Abstract] OR 'breast* milk'[Title/Abstract] OR 'human* milk'[Title/Abstract] OR 'mother* milk'[Title/Abstract] OR 'woman* milk'[Title/Abstract] OR 'women* milk'[Title/Abstract] OR ((lactating OR lactation) AND milk)[Title/Abstract]) ('Vitamin E'*[Title/Abstract] OR tocopherol[Title/Abstract]) AND (breastfed[Title/Abstract] OR breastfeed[Title/Abstract] OR breastfeeding[Title/Abstract] OR 'breast fed'[Title/Abstract] OR 'breast feed'[Title/Abstract] OR 'breast feeding'[Title/Abstract])
Web of Science	((TS=(tocopherol)) OR TS=(vitamin E)) AND ((((((TS=(breastfeeding)) OR TS=(breastfed)) OR TS=(breast feeding)) OR TS=(breast fed)) OR TS=(breastfeed)) OR TS=(breast feed)) ((TS=(tocopherol)) OR TS=(vitamin E)) AND ((((((TS=(human* milk)) OR TS=(woman*milk)) OR TS=(mother* milk)) OR TS=(breast* milk)) OR TS=(lactation)) OR TS=(lactating))
Embase	(tocopherol*:ab,ti OR 'vitaminE':ab,ti) AND ('breastmilk':ab,ti OR 'breast* milk':ab,ti OR 'human* milk':ab,ti OR 'mother* milk':ab,ti OR 'woman* milk':ab,ti OR 'women* milk':ab,ti OR ((lactating OR lactation) AND milk)) :ab,ti) (tocopherol*:ab,ti OR 'vitamin E':ab,ti) AND (breastfed:ab,ti OR breastfeed:ab,ti OR breastfeeding:ab,ti OR 'breast fed':ab,ti OR 'breast feed':ab,ti OR 'breast feeding':ab,ti)

size, relevant characteristics of mother (age, gestational weeks etc.) and data of vitamin E concentration.

Assessment of study quality

The quality of studies was assessed according to the Joanna Briggs Institute (JBI) critical appraisal checklist (8–10). This assessment tool was chosen as it has been widely used in systematic reviews.

Statistical analysis

Data conversion

Total vitamin E activity was calculated as follows (11–13):

$$\begin{aligned}\alpha - TE = {} & (mg\alpha - tocopherol \times 1.0) + (mg\beta - tocopherol \times 0.5) \\ & + (mg\gamma - tocopherol \times 0.1) + (mg\delta - tocopherol \times 0.03) \\ & + (mg\alpha - tocotrienol \times 0.3) + (mg\,\beta - tocotrienol \times 0.05).\end{aligned}$$

The vitamin E data reported in different units were converted to mg α-TE/l uniformly. For instance, millimoles could be converted to milligrams by multiplying by molecular weight. Breast milk data used per kilogram could be converted to per liter by dividing by 1.032.

Data consolidation

Data in different studies presented in non-consistent forms, such as median, minimum/maximum values, and/or quartiles. Therefore, sample mean and standard deviation were estimated to pool results in a consistent format (14, 15).

If multiple data existed in the same lactation period in one study, the weighted mean (Means) and standard deviation (SDs) could be calculated with the following formula:

$$\text{Means} = \frac{(n_1 \times M_1 + n_2 \times M_2 + n_3 \times M_3 + \cdots + n_i \times M_i)}{(n_1 + n_2 + n_3 + \cdots + n_i)}$$

$$\text{A}_\text{i} = S_i^2\,(n_i - 1) + M_i^2 \times n_i$$

$$\text{SDs} = \sqrt{\frac{\sum A_i - \frac{[\sum (M_i n_i)]^2}{N}}{N-1}}$$

Where: n_i =Sample size of individual studies, M_i =Mean of individual studies, S_i = Standard deviation of individual studies.

Meta-analysis

The meta-analysis was performed by Stata software (version 14.0). The program of "metan" was used to pool vitamin E concentration in the format of means with 95% confidence intervals (95% CIs). The I^2 and the Cochran Q test were used to assess heterogeneity. $I^2 >$ 50% was considered to have substantial heterogeneity, and the random effect model was chosen. Otherwise, the fixed-effect model was used. The publication bias was evaluated by Egger's test and trim-and-fill analysis. P-value <0.05 was considered as statistically significant. The trim-and-fill analysis was a non-parametric method for approximating the number of missing studies that might help in reducing and correcting publication bias in meta-analysis.

The possible sources of heterogeneity were identified by the multivariable meta-regression model. Subgroup

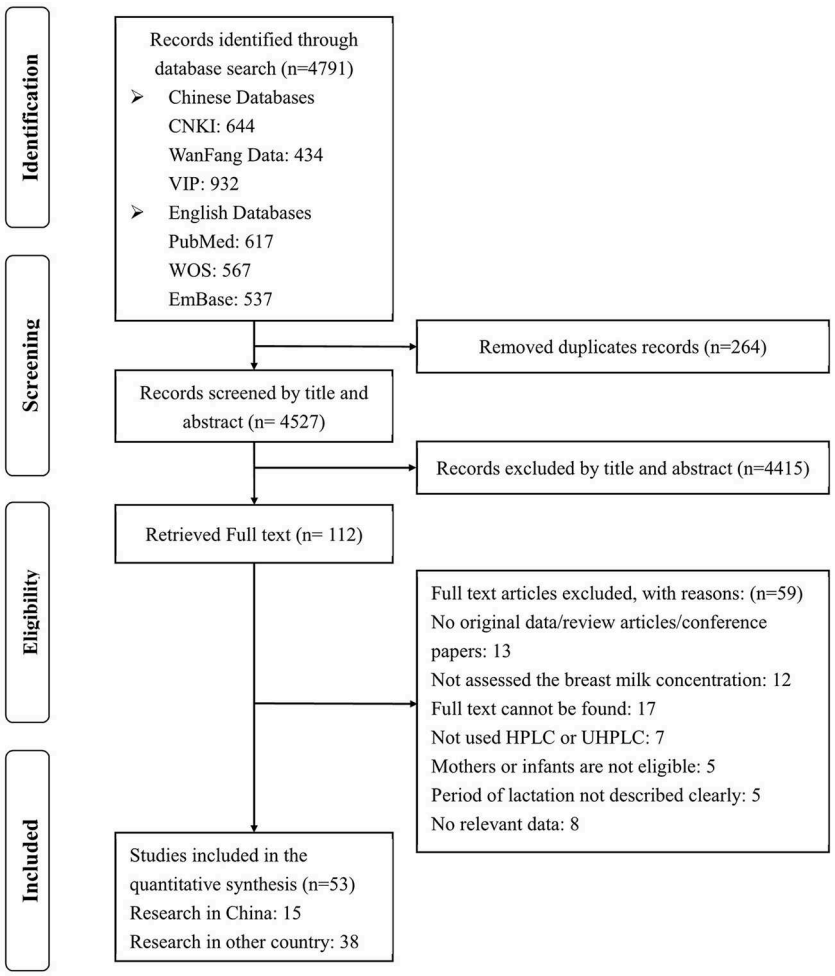

FIGURE 1
Study selection flow.

analyses were conducted based on publication year, country of study, region and research type. Sensitivity analysis was also conducted to examine the effect of every study on the final results.

Result

Data search results and included studies

Four thousand and seven hundred and ninety one studies were found from all databases. Then, 4,527 studies were reserved after duplicates were removed and 4,415 articles were excluded by checking the titles and abstracts. For the remaining 112 articles, the full texts were rigorously reviewed. After the screening, 53 papers were included in this study (Figure 1).

Study characteristics

Of the 53 included articles, 46 were descriptive studies (6, 16–60), 1 was a case-control study (61), 1 was a non-randomized controlled trial (62), and 5 were randomized controlled trials (63–67). A summary of these findings was presented in Table 2 and Supplementary Tables S1–S4.

It should be noticed that 7 studies reported vitamin E concentrations in breast milk from mothers of preterm and full-term infants both. However, the results of normal mothers who gave birth to full-term infants were used only in present study. Moreover, the data of vitamin E concentrations in healthy control group were chosen in case-control study, non-randomized controlled or randomized controlled trials.

TABLE 2 Characteristics of included studies for the meta-analysis.

Reference	Country	Region	Type of study	Colostrum		Transitional milk		Mature milk		Study quality
				Sample size	Concentration (mg α-TE/l)	Sample size	Concentration (mg α-TE/l)	Sample size	Concentration (mg α-TE/l)	
Lennart et al. (16)	Sweden	Western country	cross-sectional study	6	10.00 ± 5.50	10	4.80 ± 1.80	24	3.20 ± 1.80	Medium
Chappell et al. (17)	Canada	Western country	cross-sectional study	12	15.00 ± 2.50[a]					Medium
Chappell et al. (18)	Canada	Western country	longitudinal study	12	15.48 ± 8.80[a]			12	1.50 ± 6.34	Medium
Haug et al. (19)	Germany	Western country	longitudinal study	25	8.33 ± 9.82[a]			34	3.19 ± 1.35	Medium
Moffatt et al. (20)	America	Western country	cross-sectional study					5	3.12 ± 0.58	Medium
Boersma et al. (21)	Saint Lucia	Western country	longitudinal study	13	22.39 ± 14.3	11	13.59 ± 8.65	12	8.24 ± 4.8	High
Zheng et al. (47)	China	Asian country	cross-sectional study	43	6.94 ± 3.51[a]					Medium
Zheng et al. (48)	China	Asian country	cross-sectional study	38	3.45 ± 1.18[a]	5	1.32 ± 0.59[a]			Medium
Zheng et al. (Chinese) (49)	China	Asian country	cross-sectional study	71	5.57 ± 2.70					Medium
Barua et al. (22)	Bangladesh	Asian country	cross-sectional study					61	2.04 ± 0.86	High
Barbas et al. (23)	Spain	Western country	longitudinal study	8	14.40 ± 6.50			8	3.10 ± 1.40	Medium
Ortega et al. (24)	Brazil	Western country	longitudinal study			57	1.80 ± 0.68	57	0.96 ± 0.31	High
Zheng et al. (Chinese) (50)	China	Asian country	cross-sectional study	12	9.12 ± 1.40					High
Zheng et al. (Chinese) (67)	China	Asian country	randomized controlled trial	30	7.30 ± 3.29[b]					Medium
Macias et al. (25)	Cuba	Western country	longitudinal study	21	11.80 ± 6.30	21	5.00 ± 3.00	21	2.70 ± 1.10	High
Olafsdottir et al. (26)	Iceland	Western country	cross-sectional study					77	4.4 ± 1.85	High
Zhu et al. (Chinese) (51)	China	Asian country	longitudinal study	40	8.98 ± 3.74	40	4.47 ± 1.64	40	3.31 ± 1.13	High
Schweigert et al. (27)	Germany	Western country	longitudinal study	21	22.01 ± 13.39			21	5.69 ± 2.20	High
Sakurai et al. (28)	Japan	Asian country	cross-sectional study	6	5.95 ± 2.65	6	5.23 ± 2.67	103	2.98 ± 1.28	High
Romeu-Nadal et al. (29)	Spain	Western country	cross-sectional study					10	3.89 ± 0.16	Medium
Tokusoglu et al. (30)	Turkey	Western country	cross-sectional study					92	9.84 ± 2.13	High
Sziklai-László et al. (33)	Hungary	Western country	cross-sectional study			12	4.19 ± 2.20	18	3.12 ± 1.20	High
Grazyna et al. (31)	Poland	Western country	cross-sectional study					30	4.13 ± 1.94	High
Molto-Puigmarti et al. (32)	Spain	Western country	longitudinal study	10	37.93 ± 24.57			10	3.87 ± 2.48	Medium
Tijerina-Sáenz et al. (34)	Canada	Western country	cross-sectional study					60	2.27 ± 0.92	Medium
Orhon et al. (61)	Turkey	Western country	case-control study	20	13.27 ± 0.69[b]					High
Garcia et al. (62)	Brazil	Western country	non-randomized controlled trial	74	10.81 ± 7.42[b]					High

(Continued)

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TABLE 2 (Continued)

Reference	Country	Region	Type of study	Colostrum		Transitional milk		Mature milk		Study quality
				Sample size	Concentration (mg α-TE/l)	Sample size	Concentration (mg α-TE/l)	Sample size	Concentration (mg α-TE/l)	
Yu et al. (52)	China	Asian country	cross-sectional study	7	3.04 ± 1.94	7	1.80 ± 0.62	66	2.42 ± 1.64	High
Antonakou et al. (35)	Greece	Western country	cross-sectional study					126	3.85 ± 1.86	High
Kasparova et al. (36)	Czech Republic	Western country	cross-sectional study					48	3.83 ± 1.45	Medium
Szlagatys-Sidorkiewicz et al. (6)	Poland	Western country	longitudinal study	49	8.69 ± 5.18			49	1.94 ± 2.41	High
Martysiak-Zurowska et al. (39)	Poland	Western country	longitudinal study	17	10.13 ± 1.50	30	4.59 ± 0.93	46	2.64 ± 0.89	High
de Lira et al. (37)	Brazil	Western country	cross-sectional study	103	11.24 ± 5.51					High
Grilo et al. (38)	Brazil	Western country	cross-sectional study	71	10.94 ± 5.32[a]					High
Fang et al. (Chinese) (53)	China	Asian country	cross-sectional study	72	9.29 ± 5.33			31	2.90 ± 1.50	Medium
Clemente et al. (63)	Brazil	Western country	randomized controlled trial	72	16.54 ± 1.71[b]					High
Liu et al. (Chinese) (55)	China	Asian country	cross-sectional study	5	2.13 ± 0.91	10	2.21 ± 1.12	38	2.49 ± 1.01	High
Jiang et al. (54)	China	Asian country	longitudinal study	102	6.32 ± 4.25	102	2.56 ± 2.25	102	1.83 ± 1.12	High
Grilo et al. (64)	Brazil	Western country	randomized controlled trial	88	12.02 ± 6.78[b]			27	2.48 ± 1.01	Medium
Xue et al. (56)	China	Asian country	cross-sectional study	77	7.76 ± 6.13	89	4.25 ± 2.56	270	2.70 ± 1.78	High
Kim et al. (40)	Korea	Asian country	cross-sectional study					165	2.10 ± 1.10	High
Silva et al. (42)	Brazil	Western country	cross-sectional study	100	17.44 ± 6.46	77	5.99 ± 2.24	63	3.45 ± 1.64	High
Melo et al. (65)	Brazil	Western country	randomized controlled trial	78	15.80 ± 8.83[b]					Medium
Samano et al. (41)	Mexico	Western country	cross-sectional study					32	6.64 ± 3.2	High
Wei et al. (57)	China	Asian country	longitudinal study	103	7.50 ± 2.10	103	3.80 ± 1.40	103	3.10 ± 1.40	High
Wu et al. (Chinese) (58)	China	Asian country	longitudinal study	89	11.81 ± 5.33	89	4.69 ± 1.81	89	4.26 ± 2.05	High
de Sousa Reboucas et al. (66)	Brazil	Western country	randomized controlled trial					80	2.98 ± 0.81[b]	Medium
Machado et al. (43)	Brazil	Western country	cross-sectional study					38	0.52 ± 0.10	High
da Mata et al. (44)	Brazil	Western country	cross-sectional study					103	3.06 ± 1.70	High
Wu et al. (Chinese) (60)	China	Asian country	longitudinal study	89	9.72 ± 5.22	89	4.58 ± 1.81	89	4.23 ± 1.95	High
Wu et al. (59)	China	Asian country	longitudinal study	42	10.12 ± 4.52	42	5.35 ± 1.97	42	3.73 ± 1.63	High
Duan et al. (45)	Korea	Asian country	cross-sectional study					34	3.82 ± 1.75	High
Zagierski et al. (46)	Poland	Western country	cross-sectional study					154	3.82 ± 1.22	High

[a] Data refer to healthy mothers of full-term infants.
[b] Data refer only to the control group.

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Meta-analysis results

Results of syntheses

The pooled mean vitamin E concentration in colostrum was 10.57 mg α-TE/L (95%CI 8.94–12.20), transitional milk was 4.03 mg α-TE/L (95%CI 3.29–4.77), and mature milk was 3.29 mg α-TE/L (95%CI 2.95–3.64) (Table 3).

Forest plot showed that the minimum and maximum values of vitamin E content in colostrum were 2.13 mg α-TE/L (55) and 37.93 mg α-TE/L (32), in transitional milk were 1.32 mg α-TE/L (48) and 13.59 mg α-TE/L (21), in mature were 0.52 mg α-TE/L (43) and 9.84 mg α-TE/L (30) (Figure 2).

Heterogeneity

Publication year, region (Asia or not), country of study and research type were analyzed for the source of heterogeneity by meta-regression analysis (multivariable). Results showed region might be the source of heterogeneity in colostrum (Table 4). We provide summary estimates of vitamin E content; however, the I^2 statistic indicated that data were heterogeneous in many of our analyses and therefore these summary measures must be interpreted with appropriate caution.

Subgroup analyses

The pooled concentration of vitamin E in colostrum was 13.34 mg α-TE/L (95%CI 11.97–14.72) in Western countries (19 studies were included) and 7.18 mg α-TE/L (95%CI 5.84–8.52) in Asian countries (16 studies were included).

The result in transitional milk was 5.00 mg α-TE/L (95%CI 3.27–6.73) in Western countries (7 studies were included) and 3.61 mg α-TE/L (95%CI 2.90–4.32) in Asian countries (11 studies were included). The data in mature milk was 3.61 mg α-TE/L (95%CI 2.90–4.32) in Western countries (29 studies were included) and 2.97 mg α-TE/L (95%CI 2.59–3.35) in Asian countries (14 studies were included) (Figure 3).

Sensitivity analyses

In this review, most studies had the consistent influence on the overall estimation of meta-analysis except three articles (21, 30, 43), which had a small influence over other researches of mature milk (Figure 4).

Publication bias

The Egger's test of mature milk ($P < 0.001$) revealed evidence of publication bias. Trim-and-fill analysis estimated 12 missing studies. The overall effect measure based on this analysis was 3.98 mg α-TE/L (95%CI 3.02–4.93) (Figure 5), which was slightly higher than the originally reported overall effect measure (Figure 2C). This adjusted estimate suggested a lower risk of bias than the original analysis.

Discussion

To our knowledge, this could be the first meta-analysis that evaluated the level of vitamin E in healthy mothers at different stages of lactation and it revealed a number of interesting findings.

Colostrum milk

Colostrum, which is generated from the first day until the seventh or tenth day following parturition, is the first milk lactated (68). The publication of World Health Organization (WHO) and United Nations Children's Fund (?UNICEF)? have demonstrated that breastfeeding with colostrum milk within the first hour of new life could effectively decrease neonatal mortality. It undoubtedly highlights the significance of breastfeeding right away upon delivery (69).

It is reported that colostrum is characterized by the highest concentration of vitamin E. The significant reduction can be observed in transitional milk and mature milk. Given that the concentration of vitamin E in plasma of neonates is usually much lower than that of adults including their mothers, high vitamin E consumption from colostrum seems to provide a compensatory mechanism of antioxidative activity (6).

TABLE 3 Meta-analysis summary.

Periods of lactation	Concentration of vitamin E					
	Number of studies	Sample size	Overall effect (95% CI)	Heterogeneity test		Egger's test
				I^2 (%)	*P*	*P*
Colostrum milk	35	1,626	10.57 (8.94–12.20)	99.1	0.000	0.954
Transitional milk	18	800	4.03 (3.29–4.77)	97.8	0.000	0.063
Mature milk	42	2,562	3.39 (2.86–3.92)	99.6	0.000	0.000

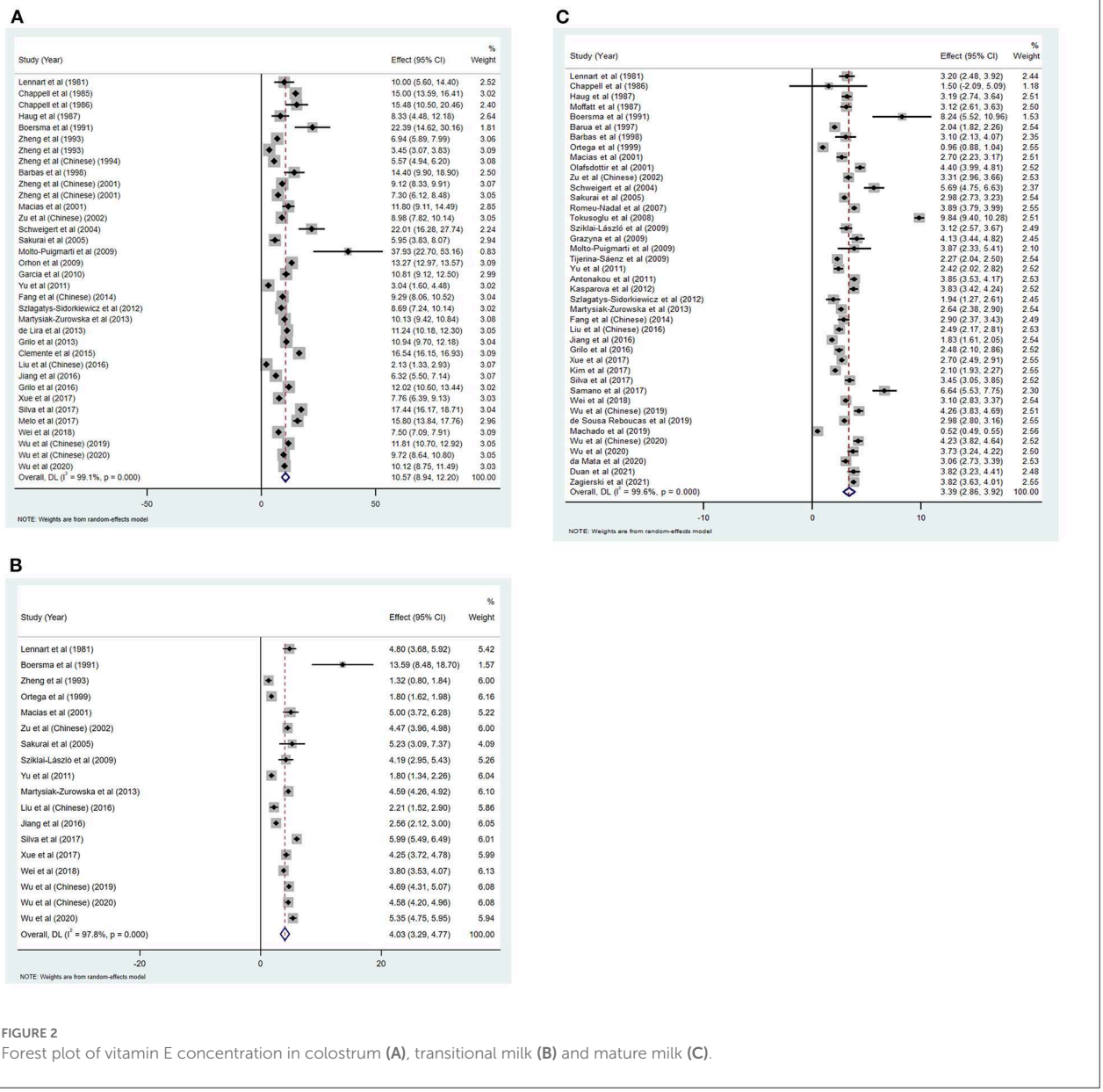

FIGURE 2
Forest plot of vitamin E concentration in colostrum **(A)**, transitional milk **(B)** and mature milk **(C)**.

TABLE 4 Meta-analysis summary.

Periods of lactation	Publication year		Country		Region		Research type	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Colostrum milk	0.079	0.200	−0.355	0.049	8.821	0.000	0.281	0.562
Transitional milk	0.023	0.679	−0.023	0.861	1.656	0.367	0.648	0.530
Mature milk	0.004	0.888	0.013	0.826	0.613	0.414	−0.217	0.477

In this study, 35 evidence demonstrated the level of vitamin E in colostrum, 18 and 42 evidence reported the vitamin E concentration in transitional milk and mature milk, respectively. The results of the meta-analysis showed vitamin E concentration was significantly higher in colostrum (10.57 mg α-TE/L) than in transitional milk (4.03 mg α-TE/L) and mature milk (3.29 mg

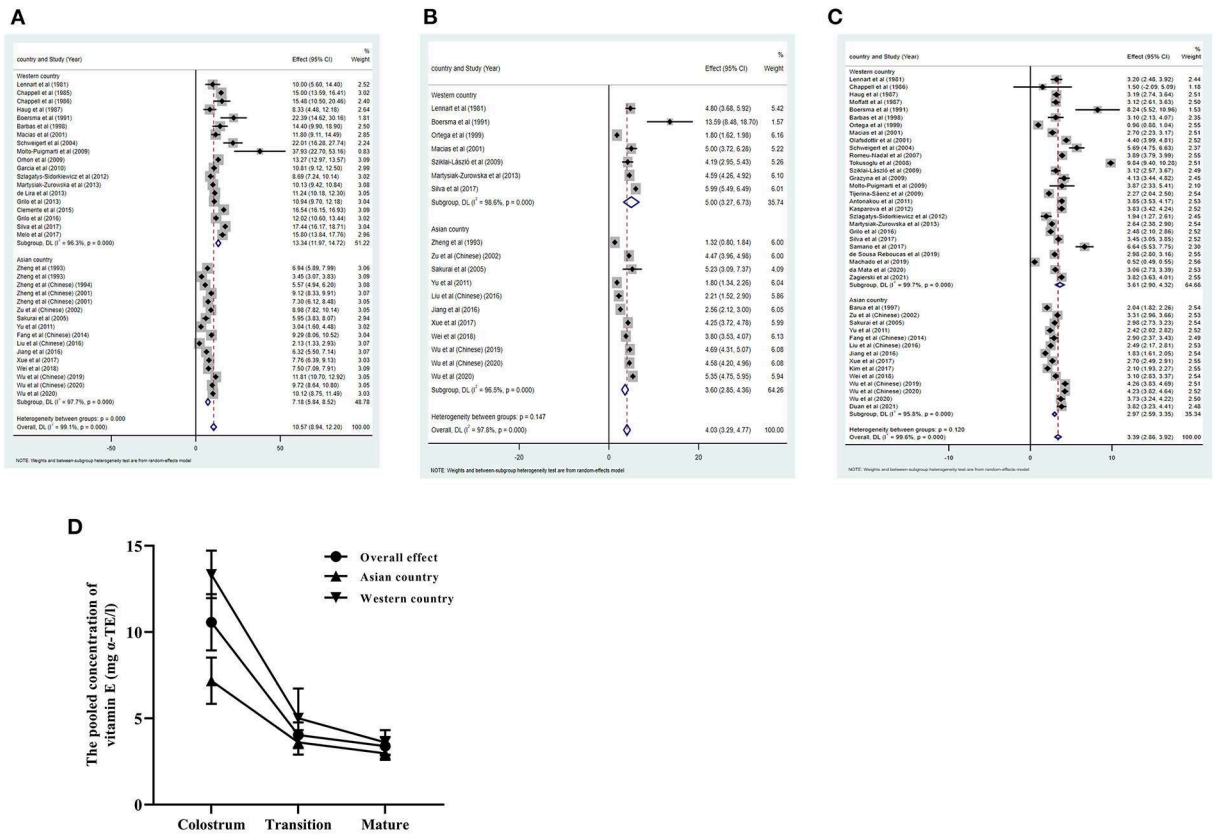

FIGURE 3
Sub-group analysis showed the pooled concentration of vitamin E based on Western countries and Asian countries in colostrum **(A)**, transitional milk **(B)** and mature milk **(C)**. The trend of pooled results in different periods of lactation **(D)**.

α-TE/L). The trend of these pooled results was in line with the longitudinal studies that reported different lactation periods (16, 21, 25, 28, 39, 42, 51, 54, 56–60). Throughout lactation, vitamin E levels decreased constantly. This vitamin E reduction in breast milk could be explained by the fact that, after the first few days of lactation, the diameter of milk fat globules increases as milk matures, and the synthesis and secretion of triglycerides increase, without a proportional increase in the secretion of phospholipids and other components (including tocopherols, cholesterol, and the percentage of long-chain PUFAs) of the membranes of fat globules (70). Therefore, there is a significant reduction in the levels of alpha-tocopherol.

The studies that were chosen included 19 researches on the colostrum of Western lactating women and 16 studies of Asian lactating women. Subgroup analyses showed that Asian women had significantly lower levels of vitamin E in their colostrum than did Western women. The reason for this difference might be discovered through comparing results between original studies. Maternal characteristics, genetic background, dietary intake of vitamin E and the use of supplementation appeared to be the main factors for the discrepancy of vitamin E level in breast colostrum between different regions (2, 57, 65, 68).

It's worth noting that a discrepancy of vitamin E concentration could be observed in different research times. In recent 10 years, colostrum are explored in 8 studies in Western lactating women. The vitamin E concentrations (16.54 ± 1.71 mg/L, 12.02 ± 6.78 mg/L, 17.44 ± 6.46 mg/L, 15.8 ± 8.83 mg/L) (42, 63–65) in the latest 4 Brazilian studies from 2015 to 2017 were higher than those in the articles from Poland (8.69 ± 5.18 mg/L, 10.13 ± 1.5 mg/L) (6, 39) and Brazil (11.24 ± 5.51 mg/L, 10.94 ± 5.32 mg/L) (37, 38) both in 2013. The same phenomenon could be found in the research of China. Moreover, vitamin E levels in colostrum also could be found regional discrepancy in China (Supplementary Figure S1). Three researches of Wu et al. (58–60) observed the vitamin E values of colostrum in Shanghai (9.72 ± 5.22 mg/L, 10.12 ± 4.52 mg/L, 11.81 ± 5.33 mg/L) from 2019 to 2020 were much higher than Inner Mongolia (3.04 ± 1.94 mg/L) in 2009 (52), Hohhot (2.13 ± 0.91 mg/L) in 2013 (55), Hangzhou (4.40 ± 2.85 mg/L) in 2016 (54), Lanzhou (8.09 ± 4.85 mg/L) in

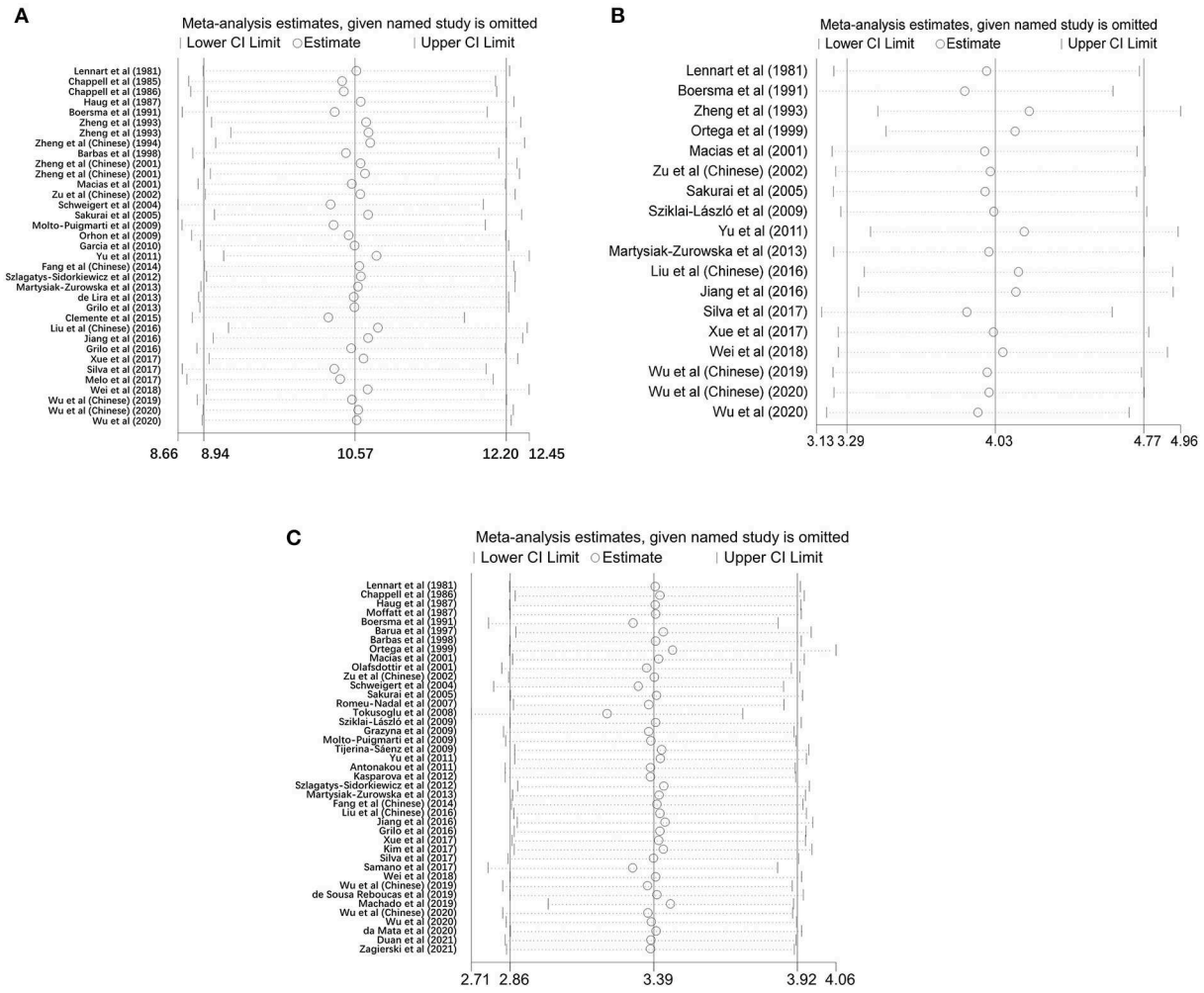

FIGURE 4
Sensitivity analysis showed the influential studies on the overall pooled effect for colostrum **(A)**, transitional milk **(B)** and mature milk **(C)**.

2016 (54), Beijing (6.53 ± 4.12 mg/L) in 2016 (54). The reason might be associated with the improved economic conditions and increased breastfeeding health awareness. Improving the nutritional status of breastfeeding mothers has an extremely important impact on the ideal breast milk of lactating mothers.

Transitional milk

The composition of milk gradually changes after childbirth. Breast milk produced from the eighth to the fifteenth day after delivery was known as transitional milk (68). The vitamin E concentration in transitional milk was lower than that in colostrum but higher than that in mature milk, which is similar to other researches (16, 21, 24, 25, 28, 33, 39, 42, 51, 54, 56–60).

The subgroup analyses observed that vitamin E concentration in transitional milk of Western lactating women was higher than that of Asian lactating women. Other important factors must be taken into account in addition to dietary restrictions and ethnicity. We found that the collection time of transitional milk was inconsistent in various studies. For example, the transitional milk is collected from the 21st to the 24th day postpartum (51), or the 8th to the 21th day postpartum (52, 55) in several prior studies in China. However, the latest studies in China (54, 56–60) revealed the collection time is from the 5th to 15th day postpartum, which is comparable to the majority of studies conducted in other nations (16, 21, 24, 25, 33, 39, 42). It might be an important reason resulting in the lower pooled vitamin E concentration of transitional milk in Asian countries. In order to increase the reliability of the results, more researches of transitional milk collected from the 5th to 15th day postpartum are needed.

Moreover, in a study of Saint Lucia (21), the result of vitamin E concentration in transitional milk was much higher

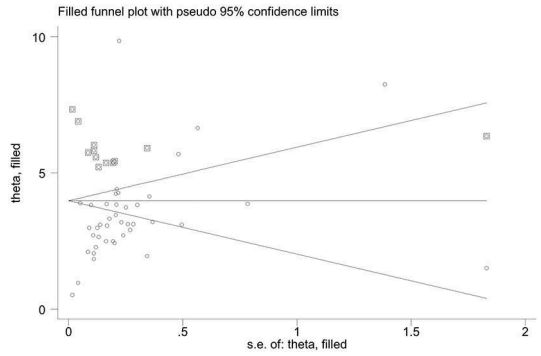

FIGURE 5
Trim-and-fill analysis estimated the number of possible missing studies for the vitamin E concentration in mature milk. The squares represented the possible missing studies.

(approximately two to three times) than that of other Western countries. However, the author did not mention the reason for this unusually high concentration. Due to the lack of transitional milk studies, the overall effect of meta-analysis of Western countries was 4.36 (95%CI 2.62–6.14) after excluding this abnormal value. One thing worth noting is the exclusion could cause a big discrepancy in results. Therefore, more data is needed to support the values as well.

Mature milk

After transition milk, variations in the composition of breast milk continue to occur, until third week postpartum. During this period, the composition of milk becomes more stable, which is mature milk (68).

The concentration of vitamin E in mature breast milk samples from Western countries were near to the values of Asian samples. It was speculated that individual or dietary factors might have little influence on mature breast milk. This speculation was supported by a study, which demonstrated maternal supplementation with R, R, R, α-tocopherol could increase vitamin E level of colostrum and transitional milk rather than mature milk (71). It is worth noting that infants with an estimated daily intake of 780 mL/day may not get enough vitamin E from mature milk to meet their nutritional needs (42, 56). Consequently, the implementation of procedures to increase the level of vitamin E in milk would be important especially for nursing mothers living in poor conditions of food safety.

Furthermore, Xue et al. (56) study the vitamin E concentration of breast milk during 12–240 day postpartum. It was found that the concentration of vitamin E in breast milk observed in 12–30th day postpartum (2.96 ± 2.11 mg/L) were similar to those collected in 31th-240th day postpartum (31–60th day: 2.96 ± 1.92 mg/L, 61–120th day: 2.45 ± 1.67 mg/L, 121–240th day: 2.71 ± 1.72 mg/L). It could be implied that vitamin E concentration in breast milk might reach a relatively stable level after 12th day postpartum. Another study observed in Japan in 2005 (28) showed that vitamin E concentration in breast milk in 21–89th day postpartum (2.97 ± 1.23 mg α-TE/L) were same as those in 90–180th day and 181–365th day postpartum (3.45 ± 1.39 mg α-TE/L and 2.52 ± 1.03 mg α-TE/L). The plateau of vitamin E concentration in breast milk appeared almost one week later than the result of Xue et al. It is speculated that the vitamin E of breast milk continues to decrease after childbirth, until approximately second to third week postpartum. The vitamin E concentration in mature milk becomes more stable. However, to support the start of the vitamin E stationary phase, more evidence should be done.

One Turkish study in 2008 (30) showed that the content of vitamin E in mature milk (9.84 ± 2.13mg/L) was significantly greater (more than three times) than the samples from other nations such as Greece (35) and Spain (32). As a result, the statistical data of this paper may be influenced by the potential confounders. According to a Brazil study in 2019, the α-tocopherol content of breast milk was only 0.56 ± 0.11mg/L from 17th to 28th days postpartum, significantly lower than other studies conducted there during the same time period (44, 66). It may be associated with lower sample size.

Limitation

There were some limitations to our study. First, the search was restricted to the studies published in English language and Chinese language, which may lead to publication bias. However, we have addressed the issue of publication bias during our analysis. Next, although subgroup and sensitivity analyses were performed, heterogeneity was still very large in the meta-analysis. Except for differences in region may lead to greater heterogeneity between studies especially in colostrum, the other factors could also contribute to heterogeneity. The underlying factors, including milk sample collection method, different techniques for nutrient measurements, postpartum milk sampling, time of milk sampling, duration of breastfeeding and so forth, might partly explain the large variation between studies in different periods of lactation. Therefore, more studies are necessary for reliable results.

Conclusion

Vitamin E concentration in breast milk decreased during lactation until the mature milk was produced. The higher value of vitamin E in colostrum might be important for

new-borns to defend early oxidative stress. The vitamin E concentration in colostrum from western countries was higher than from Asia, which might be related to dietary habits, individual variation, etc. More evidences of vitamin E concentration in transitional milk, especially the milk collected from the 5 to 15th day postpartum, are needed. The vitamin E content of mature milk was similar. It tended to be stable from about second week postpartum to 4–6th month postpartum. More results are needed to support this conclusion.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

YX, HZ, and AZ: applied the literature search and undertook the screening title and abstract screening. XW and XR: extracted the data and tabulated results. KL, YX, and XW: statistical analysis. YX and XW: wrote the initial version of the manuscript. YY: validation of the paper for important figures. JL and RX: critical revision of the paper for important intellectual content. All authors contributed to its final version and read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1050011/full#supplementary-material

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Promotion effect of the blend containing 2′-FL, OPN and DHA on oligodendrocyte progenitor cells myelination *in vitro*

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During early neurodevelopment of infant, myelination plays an essential role in brain connectivity and emergence of behavioral and cognitive function. Early life nutrition is an important factor to shape myelination and consequently cognitive appearance. To analyze the effects of additive nutrients, including 2′-fucosyllactose (2′-FL), osteopontin (OPN), docosahexaenoic acid (DHA), on neurocognitive function and brain structure, the current study evaluated the effects of different composition of breast milk nutrients on oligodendrocyte progenitor cells (OPCs) myelination with a neural primary cell model *in vitro*. The study showed that the three nutrients promoted the proliferation, maturation and differentiation of OPCs into mature oligodendrocytes (OLs) in each phage of the cell growth, and the effect of the nutrients blend is obviously stronger than that of the nutrient treatment alone, showing a synergistic effect in promotion of OPCs. The results of this experiment clarified the effects of 2′-FL OPN and DHA to promote myelination development of neural cells, and laid an experimental basis for further optimization of infant formula.

KEYWORDS

myelination, 2′-fucosyllactose, osteopontin, docosahexaenoic acid, neural primary cell

Introduction

Early life nutrition plays a critical role in neurodevelopmental processes such as neuronal maturation, synaptogenesis, and myelination (1). Myelination is the process of specialized glial cells oligodendrocytes (OLs) in the central nervous system (CNS), to form myelin sheaths around axons, which is essential for normal brain cells connectivity (2, 3). Myelin sheath, composed of several condensed lipid bilayer membranes (4), increases axonal conduction velocity and the maturation of cognitive function by reducing the capacitance of the axonal membrane and allows jumping currents (5–7). Oligodendrocyte progenitor cells (OPCs) are the main glial cell population in the central nervous system, accounting for 5–8% of the total cell population (8). Post-mitotic

OPCs differentiate into myelinated OLs, and these OLs expand a number of processes, establishing contacts with axons of different neurons and initiating myelination (9, 10). During their maturation, OLs produce different components of myelin as lipids (cholesterol, galactolipids, and phospholipids) and myelin-specific proteins. The types of myelin proteins expressed by OLs, such as myelin-associated glycoprotein (MAG) and myelin basic protein (MBP), closely correlate with its maturity (11, 12). Myelinated OLs express MAG, and the expression of MAG gradually increases during the maturation of OLs. MAG is a sialic acid-binding immunoglobulin-like lectin, and although it constitutes only a small fraction of the total protein content of myelin, it is predominantly expressed in the peri-axonal region of myelin (13). It appears to play an important role in oligodendrocyte-axon interactions and mediate bidirectional signaling between axons and OLs to support myelination (14). MBP is expressed in mature myelinated OLs and is one of the main components of myelin. MBP appears to play an active role in myelination and compaction. In fact, MBP aggregates and forms a cohesive reticulin network, which is essential for hopping currents (15, 16).

In the CNS, every step of myelination, including the proliferation of OPCs, the differentiation and maturation of OPCs into myelinating OLs, and myelination, is highly regulated by both external and internal factors. In particular, different nutrients have different effects on myelination, suggesting that early life nutrition may have important implications for the regulation of myelination. Therefore, identifying early-life nutritional factors that support myelination is critical for optimal brain and cognitive development.

Osteopontin (OPN), 2′-fucosyllactose (2′-FL), and docosahexaenoic acid (DHA) are essential nutrients in breast milk and infant formula. Many studies have proved that OPN plays an important role in organism, especially in the process of immune activation, bone damage repair, vascular regeneration and bone remodeling (17). 2′-FL, a breast milk oligosaccharide, possesses physiological functionalities of prebiotics effect, antiadhesive antimicrobials, immunomodulation and promotion of brain development (18). DHA is a key nutritional n-3 PUFA and was found to have a strong influence on brain health (19). Though having promotion on neurocognitive function and brain structure, the underlying mechanism of the three essential nutrients on development of neural cells remains unknown by now. In the current study, an *in vitro* model of primary cell cultures containing neurons and OLs was used to evaluate the effects of a composition of breast milk nutrients on myelination. We hoped to add nutrients to mixed cell cultures to promote the proliferation, maturation and differentiation of OPCs into mature OLs and/or the myelinating properties of OLs. The density of OPCs was firstly evaluated after 12 days of *in vitro* culture. Then, OPCs differentiation into OLs and OLs maturation and myelination were assessed by quantifying MAG-positive cells and MBP-positive cells at 18 and 30 days, respectively.

Methods, materials, and instruments

Materials and reagents

2′-FL, OPN and DHA were purchased from Beijing Jinkangpu Food Science & Technology Co., Ltd (Beijing, China). Neural cell culture medium was obtained from GibcoTM (Life Technologies Inc., Grand Island, NY, USA). A2B5 antibody (Lot. MAB312RX) and MAG antibody (Lot. MAB1567) were got from Merck Co., Inc., (NJ, USA). MBP antibody (Lot. NBP1-05204) was obtained from Novus Biologicals.

Neural primary cell acquisition

The animal experiment was approved by the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). To get primary mixed cultures of neurons and OLs (20), forebrains of neonatal rat were taken out on ice and trypsinized for 20 min at 37°C (Trypsin EDTA 1X, PAN BIOTECH). The reaction was stopped by the addition of Dulbecco's modified Eagle's medium (DMEM, PAN BIOTECH) containing DNAase II (0.1 mg/ml, PAN BIOTECH) and 10% fetal bovine serum (FCS, GIBCO). Cells were mechanically dissociated three times by 10 ml pipette and centrifuged at 515 g for 10 min at 4°C, and then were seeded in plates (2×10^4 cells/well) pre-coated with poly-L-lysine (BD Falcon) and laminin (Sigma) in a humidified incubator. The medium consisted of Neurobasal (GIBCO) supplemented with 2% B27 (GIBCO), 2 mM L-glutamine (L-Glu, PAN BIOTECH), 2% P/S solution (PAN BIOTECH), 1% FCS and 10 ng/ml of platelet-derived growth factor (PDGF-AA, PAN BIOTECH).

Neural cell culture

Cells were seeded in 48-well-plates at a density of 2×10^4 cells/well and added mix or individual nutrients in fresh medium 6 h later. The incubations were replaced with half of the medium containing the same mix or individual nutrients every other day, and stop at 12, 18, or 30 days for immunohistochemistry analysis.

Immunohistochemistry assay

Immunocytochemistry was carried out as previous report (20) with minor modification. After incubation with the nutrients for 12, 18 and 30 days, the cells were fixed with a cold mixture of 95% ethanol and acetic acid (5%) for 5 min. Non-specific sites were then blocked with 0.1% saponin (Sigma) and 1% FCS (GIBCO) in PBS for 15 min at room temperature.

At 12 days, the cells were incubated with mouse monoclonal anti-A2B5 conjugated Alexa fluor 488 (1/200, MAB312RX) in

TABLE 1 Groups and dosages of the nutrition.

Groups	Nutrition composition	2-FL(mg/ml)	OPN(mg/ml)	DHA(mg/ml)
Control	Vehicle	0	0	0
Positive	Olesoxime	0	0	0
1	2-FL(H^a)	10	0	0
2	OPN(H^a)	0	1	0
3	DHA(H^a)	0	0	5
4	2-FL(L^b)+OPN(L^b)	0.1	0.01	0
5	2-FL(L^b)+OPN(H^a)	0.1	1	0
6	2-FL(M^c)+OPN(M^c)	1	0.1	0
7	2-FL(H^a)+ OPN(L^b)	10	0.01	0
8	2-FL(H^a)+OPN(H^a)	10	1	0
9	2-FL(LM^d)+OPN(LM^d)	0.5	0.05	0
10	2-FL(MH^e) +OPN(MH^e)	5	0.5	0
11	2-FL(L^b)+OPN(L^b)+DHA(L^b)	0.1	0.01	0.05
12	2-FL(L^b)+OPN(M^c)+DHA(M^c)	0.1	1	0.5
13	2-FL(H^a)+OPN(H^a)+DHA(H^a)	10	1	5
14	2-FL(LM^d)+OPN(LM^d)+DHA(LM^d)	0.5	0.05	0.25
15	2-FL(MH^e)+OPN(MH^e)+DHA(MH^e)	5	0.5	2.5

a, high dose; b, low dose; c, medium dose; d, low to medium dose; e, medium to high dose.

TABLE 2 Effects of different nutritional compositions containing 2′-FL, OPN and/or DHA on the number of A2B5 positive cells.

Groups	Nutrition composition	Density of A2B5 positive cells (Mean ± SEM)
Control	Vehicle	77.333 ± 5.181
Positive	Olesoxime	113.833 ± 4.708***
1	2-FL(H^a)	102.167 ± 6.036**
2	OPN(H^a)	95.50 ± 4.595*
3	DHA(H^a)	100.333 ± 5.327*
4	2-FL(L^b)+OPN(L^b)	85.833 ± 5.009
5	2-FL(L^b)+OPN(H^a)	101.00 ± 7.832**
6	2-FL(M^c)+OPN(M^c)	114.50 ± 8.563$^{***\wedge\triangledown}$
7	2-FL(H^a)+ OPN(L^b)	110.333 ± 4.256***
8	2-FL(H^a)+OPN(H^a)	122.667 ± 5.823$^{***\wedge\wedge\triangledown\triangledown\triangledown}$
9	2-FL(LM^d)+OPN(LM^d)	108.167 ± 5.023***
10	2-FL(MH^e)+OPN(MH^e)	116.00 ± 4.531$^{***\wedge\triangledown\triangledown}$
11	2-FL(L^b)+OPN(L^b) + *DHA*(L^b)	92.667 ± 5.155*
12	2-FL(L^b)+OPN(M^c) + *DHA*(M^c)	114.50 ± 6.474$^{***\triangledown}$
13	2-FL(H^a)+OPN(H^a) + *DHA*(H^a)	130.167 ± 8.867$^{***\wedge\wedge\triangledown\triangledown\triangledown\#\#}$
14	2-FL(LM^d)+OPN(LM^d) + *DHA*(LM^d)	107.667 ± 7.315**
15	2-FL(MH^e)+OPN(MH^e) + *DHA*(MH^e)	128.50 ± 8.086$^{***\wedge\wedge\triangledown\triangledown\triangledown\#\#}$

*$p < 0.05$ compared with blank control group, **$p < 0.01$ compared with blank control group, $^{***}p < 0.001$ compared with blank control group.
$^\wedge$Compared with 2′-FL high-dose group $p < 0.05$, $^{\wedge\wedge}$compared with 2′-FL high-dose group $p < 0.01$.
$^\triangledown$Compared with OPN high-dose group $p < 0.05$, $^{\triangledown\triangledown}$ compared with OPN high-dose group $p < 0.01$, $^{\triangledown\triangledown\triangledown}$ compared with OPN high-dose group $p < 0.001$.
$^{\#\#}$ Compared with DHA high-dose group $p < 0.01$.
a, high dose; b, low dose; c, medium dose; d, low to medium dose; e, medium to high dose.

PBS containing 1% FCS and 0.1% saponin for 2 h at room temperature. After washing with PBS for 3 times, the cells were incubated with a rabbit anti-neurofilament antibody (1/500, N4142) PBS containing 1% FCS and 0.1% saponin for 2 h at room temperature. Neurofilament (NF) was stained with a secondary goat anti-rabbit CF568 antibody (1/400, SAB4600084,

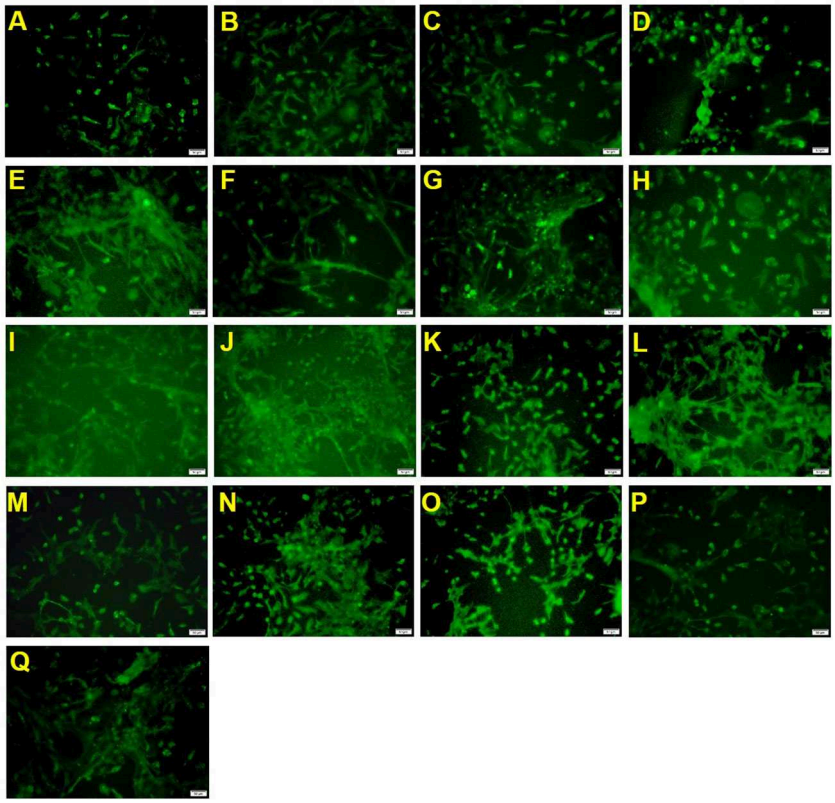

FIGURE 1
A2B5 immunostaining of neural primary cells after treatment with 2′-FL and OPN and DHA for 12 days. **(A)**, control group; **(B)**, positive drug; **(C)**, 2′-FL(H); **(D)**, OPN(H); **(E)**, DHA(H); **(F)**, 2′-FL(L)+OPN(L); **(G)**, 2′-FL(L)+OPN(H); **(H)**, 2′-FL(M)+OPN(M); **(I)**, 2′-FL(H)+OPN(L); **(J)**, 2′-FL(H)+OPN(H); **(K)**, 2′-FL(LM)+OPN(LM); **(L)**, 2′-FL(MH) +OPN(MH); **(M)**, 2′-FL(L)+OPN(L)+DHA(L); **(N)**, 2′-FL(L)+OPN(M)+DHA(M); **(O)**, 2′-FL(H)+OPN(H)+DHA(H); **(P)**, 2′-FL(LM)+OPN(LM)+DHA(LM); **(Q)**, 2′-FL(MH)+OPN(MH)+DHA(MH). Scale bar: 50 μm.

SIGMA) containing 1% FCS and 0.1% saponin in PBS for 1 h at room temperature.

At 18 days, the cells were incubated with a mouse monoclonal Anti-MAG (1/400, MAB1567, Millipore) and rabbit anti-NF antibody (1/500, N4142, SIGMA) in PBS containing 1% FCS and 0.1% saponin for 2 h. After washing with PBS for 3 times, the cells were incubated with a secondary goat anti-mouse CF488A antibody (1/400, SAB4600042, SIGMA) and goat anti-rabbit CF 568 antibody (dilution: 1/400, SIGMA, SAB4600084) in PBS containing 1% FCS and 0.1% saponin at room temperature for 1 h. For all conditions, the cell nuclei were stained using a Hoechst solution (SIGMA, B1155).

At 30 days, the cells were incubated with a mouse monoclonal anti-MBP (1/1000, NBP1-05204, NOVUS) and a rabbit anti-Neurofilament antibody (1/500, N4142, SIGMA) in PBS containing 1% FCS and 0.1% saponin for 2 h. After washing with PBS for 3 times, the cells were incubated with goat anti-mouse CF488A antibody (1/800, SAB4600042, SIGMA) and goat anti-rabbit CF568 antibody (1/400, SAB4600084, SIGMA) in PBS containing 1% FCS and 0.1% saponin at room temperature for 1 h.

Microscopic analysis

Digital images were collected at 20x magnification using ImageXpress equipped with LED lights (excitation 360/480/565 and emission 460/535/620). All images were acquired with the same settings. The number of OPCs was calculated by quantifying the number of A2B5-expressing cells at 12 days, and the results were expressed as the average number of A2B5-expressing cells per well. Differentiation of OPCs to OLs was assessed by counting the number of MAG-positive cells at 18 days. Results are expressed as the average number of cells per well. At 30 days, the maturity of OLs was estimated by counting the number of MBP-positive cells.

Cell experiment grouping and dosages

To evaluate the function of nutrients in promoting the proliferation, maturation and differentiation of neuronal OPCs into mature OLs and/or myelination of OLs, the effects of different doses of nutrients mixture were studied using the

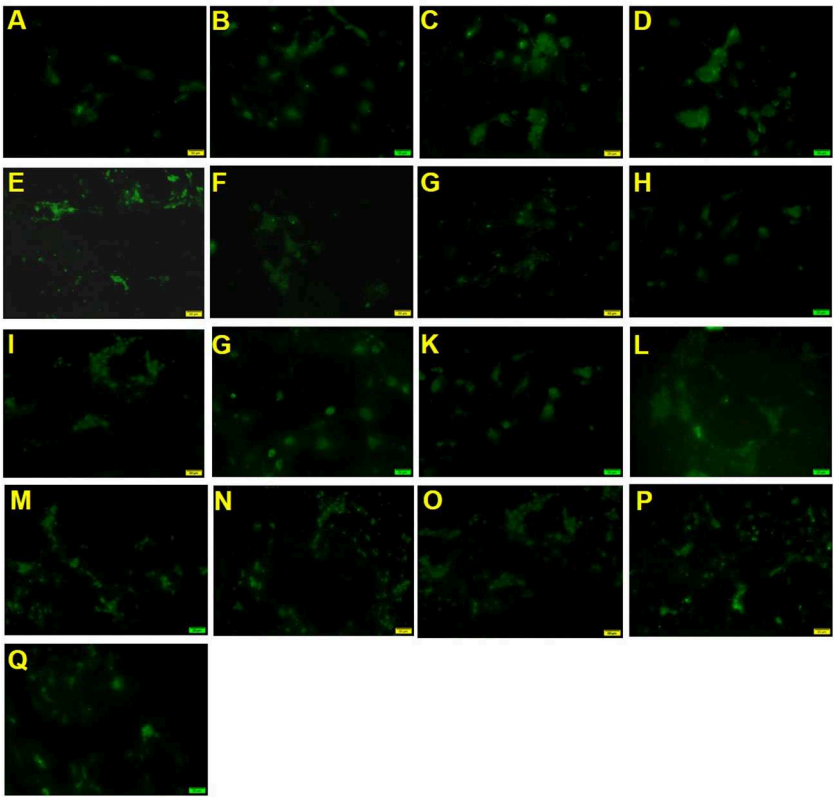

FIGURE 2
MAG immunostaining of neural primary cells after treatment with 2′-FL and OPN and DHA for 18 days. **(A)**, control group; **(B)**, positive drug; **(C)**, 2′-FL(H); **(D)**, OPN(H); **(E)**, DHA(H); **(F)**, 2′-FL(L)+OPN(L); **(G)**, 2′-FL(L)+OPN(H); **(H)**, 2′-FL(M)+OPN(M); **(I)**, 2′-FL(H)+OPN(L); **(J)**, 2′-FL(H)+OPN(H); **(K)**, 2′-FL(LM)+OPN(LM); **(L)**, 2′-FL(MH) +OPN(MH); **(M)**, 2′-FL(L)+OPN(L)+DHA(L); **(N)**, 2′-FL(L)+OPN(M)+DHA(M); **(O)**, 2′-FL(H)+OPN(H)+DHA(H); **(P)**, 2′-FL(LM)+OPN(LM)+DHA(LM); **(Q)**, 2′-FL(MH)+OPN(MH)+DHA(MH). Scale bar: 50 μm.

isolated primary neuronal cells (Table 1). The density of OPCs, OPCs differentiation into OLs and OLs maturation, and level of myelination were assessed after 12, 18, and 30 days of *in vitro* culture, respectively.

Statistical analysis

The results were expressed as mean ± standard error (mean ± SEM), and SPSS software (version 26.0, IBM, Armonk, NY, USA) was used for *T*-test and one-way ANOVA test. A value of $P < 0.05$ was considered statistically significant, while it was judged to be extremely significant when $p < 0.01$.

Results and discussion

Pro-proliferative effect of the nutrients on OPCs

To measure the effect of mixed nutrition or nutrient treatment alone on OPCs, the number of A2B5-labeled positive cells were assessed after 12 days to estimate the number of OPCs.

The sample processing results (Table 2 and Figure 1) showed that the positive drug olesoxime increased the number of A2B5 positive cells compared to the control group. 2′-FL, OPN and DHA high-dose groups (groups 1, 2 and 3) could also significantly increase the number of A2B5 positive cells, indicating that the three components contributed to the proliferation of oligodendrocyte precursor cells. However, the 2′-FL+ OPN and 2′-FL+ OPN + DHA low dose groups (groups 4 and 11) failed to increase the number of A2B5 positive cells, which suggested that the low-dose group set could not effectively induce the proliferation of oligodendrocyte precursor cells. The effects of 2′-FL + OPN middle dose group (group 6), 2′-FL + OPN high dose group (group 8), 2′-FL + OPN middle to high dose group (group 10), and 2′-FL + OPN + DHA high dose group (group 13) and 2′-FL + OPN + DHA medium to high dose group (group 15) were all significantly higher than those of the high dose groups of 2′-FL, OPN or DHA (groups 1, 2 and 3), indicating that these combinations have a synergistic effect on the growth of OPCs.

TABLE 3 Effects of different nutritional compositions containing 2′-FL, OPN and/or DHA on the number of MAG positive cells.

Groups	Nutrition composition	Density of MAG positive cells (Mean ± SEM)
Control	Vehicle	29.333 ± 3.612
Positive	Olesoxime	62.00 ± 5.520***
1	2-FL(H^a)	51.167 ± 4.624*
2	OPN(H^a)	52.333 ± 4.944*
3	DHA(H^a)	47.50 ± 6.999*
4	2-FL(L^b)+OPN(L^b)	43.00 ± 5.323*
5	2-FL(L^b)+OPN(H^a)	55.167 ± 7.213**
6	2-FL(M^c)+OPN(M^c)	60.167 ± 7.441***
7	2-FL(H^a)+ OPN(L^b)	56.00 ± 5.422**
8	2-FL(H^a)+OPN(H^a)	67.833 ± 7.305***^▽#
9	2-FL(LM^d)+OPN(LM^d)	52.833 ± 4.045**
10	2-FL(MH^e) +OPN(MH^e)	69.00 ± 7.878***^▽#
11	2-FL(L^b)+OPN(L^b)+DHA(L^b)	46.50 ± 6.402*
12	2-FL(L^b)+OPN(M^c)+DHA(M^c)	59.167 ± 7.596**
13	2-FL(H^a)+OPN(H^a)+DHA(H^a)	73.833 ± 9.250***^▽##
14	2-FL(LM^d)+OPN(LM^d)+DHA(LM^d)	57.167 ± 6.838**
15	2-FL(MH^e)+OPN(MH^e)+DHA(MH^e)	72.00 ± 9.926***^▽#

*$p < 0.05$ compared with blank control group, **$p < 0.01$ compared with blank control group, ***$p < 0.001$ compared with blank control group.
^ Compared with 2'-FL high-dose group $p < 0.05$.
▽Compared with OPN high-dose group $p < 0.05$.
Compared with DHA high-dose group $p < 0.05$, ## Compared with DHA high-dose group $p < 0.01$.
a, high dose; b, low dose; c, medium dose; d, low to medium dose; e, medium to high dose.

Nutrition promote differentiation of OPCs into mature OLs

To measure the effect of mixed nutrition or nutrition treatment alone on the myelination of OPCs, we assessed the number of MAG-labeled positive cells after treatment for 18 days.

As shown in Figure 2 and Table 3, the positive drug olesoxime showed an increase in the number of MAG-positive cells compared to the control group. The 2′-FL OPN and DHA groups (groups 1, 2 and 3) could significantly increase the number of MAG-positive cells, indicating that both components contributed to the differentiation of OPCs into mature OLs. In addition, MAG-positive cells in 2′-FL + OPN middle dose group (group 6), 2′-FL + OPN high dose group (group 8), 2′-FL + OPN middle to high dose group (group 10), and 2′-FL + OPN + DHA high dose (group 13) and 2′-FL + OPN + DHA medium to high dose group (group 15) group were all significantly higher than those of the high dose groups of 2′-FL, OPN and DHA (groups 1, 2 and 3), which suggesting that the combinations of nutrition have synergistic effect to promote differentiation of OPCs into mature OLs.

Nutrition promotes maturation and myelination of OPCs

To measure the effect of mixed nutrient or nutrient treatment alone on the cell maturation and myelination of OPCs, we assessed the number of positive cells labeled MBP after treatment for 30 days.

According to Figure 3 and Table 4, the positive drug olesoxime increased the number of MBP-positive cells compared with the blank control group. The 2′-FL, OPN and DHA groups (groups 1, 2 and 3) also could significantly increase the number of MBP-positive cells, which suggests that the components contributed to the maturation of OPCs. For mixed nutrition, 2′-FL + OPN high dose group (group 8), 2′-FL + OPN middle to high dose group (group 10), 2′-FL low dose + OPN medium dose + DHA medium dose (group 12), 2′-FL + OPN + DHA high dose (group 13), and 2′-FL + OPN + DHA medium to high dose (group 15) were significantly higher than 2′-FL, OPN or DHA single dose, indicating that these combinations have synergistic effect in maturation and myelination promotion of OPCs.

Human milk oligosaccharide (HMO) is one of the main components of human milk carbohydrates, which is closely related to the health benefits and nutrition of breastfed

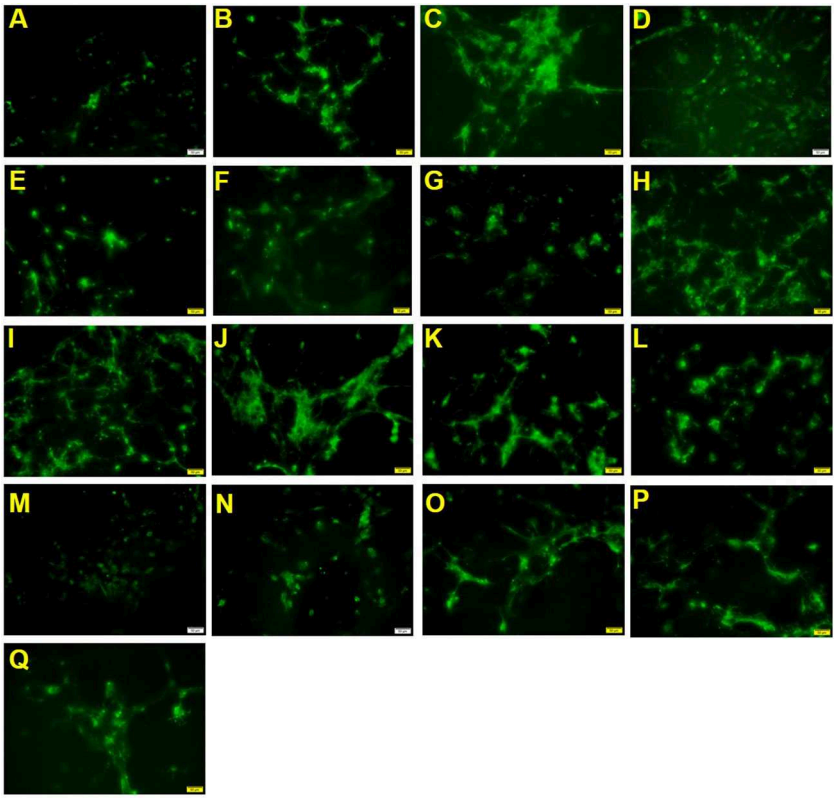

FIGURE 3
MBP immunostaining of neural primary cells after treatment with 2′-FL and OPN and DHA for 30 days. **(A)**, control group; **(B)**, positive drug; **(C)**, 2′-FL(H); **(D)**, OPN(H); **(E)**, DHA(H); **(F)**, 2′-FL(L)+OPN(L); **(G)**, 2′-FL(L)+OPN(H); **(H)**, 2′-FL(M)+OPN(M); **(I)**, 2′-FL(H)+OPN(L); **(J)**, 2′-FL(H)+OPN(H); **(K)**, 2′-FL(LM)+OPN(LM); **(L)**, 2′-FL(MH) +OPN(MH); **(M)**, 2′-FL(L)+OPN(L)+DHA(L); **(N)**, 2′-FL(L)+OPN(M)+DHA(M); **(O)**, 2′-FL(H)+OPN(H)+DHA(H); **(P)**, 2′-FL(LM)+OPN(LM)+DHA(LM); **(Q)**, 2′-FL(MH)+OPN(MH)+DHA(MH). Scale bar: 50 μm.

infants (21). As the most abundant fucosylated HMO, 2'-FL possesses various beneficial health effects as suppressing pathogen infection, regulating intestinal flora, and boosting immunity, making it has remarkable value in nutrition and medicine (22–25). Additionally, it was verified that the intake of 2′-FL affects the cognitive domain and improves the learning and memory ability of rodents (26). Thus, 2′-FL has lots of beneficial health activities. Due to its various physiological and biological effects, 2′-FL has been assessed and authorized as a new food additive to many foods. OPN is an acidic and highly phosphorylated glycoprotein, and expressed in a variety of tissues including liver, skeletal muscle, brain, and mammary gland (27, 28). Previous studies revealed that OPN is significantly involved in immunity system development and regulation. Evidence indicated that OPN plays a key role in some autoimmune, cancer and cardiovascular disease (29–31). Recently, further researches revealed an important function of OPN involving in the regulation of myelination in central nervous system (32, 33). OPN is relatively resistant to digestion, and orally ingested OPN can be absorbed into the circulatory system (33), therefore, making it plays essential roles in the development in early life and an ideal milk powder additive. DHA is well-known for its effects in intellectual development in early life. Although it plays a key role in the growth and maturation of the infant's brain, DHA cannot be synthesized efficiently in the body (34). During rapid phases of brain growth, large amounts of DHA is needed. Thus, adding DHA to milk powder as an external source has become an inevitable choice when the supply of breast milk is insufficient.

Although 2′-FL, OPN and DHA are important for cognitive development as the milk powder additives, the effect of the three components for neuronal maturation, synaptogenesis, and myelination still little is known. In this study, we evaluated the effect of the three nutrients on promoting the myelination, including the proliferation of OPCs, the differentiation and maturation of OPCs into myelinating OLs, of primary neuronal cells *in vitro*. The results showed that the three components contributed to the proliferation, differentiation, maturation and myelination of OPCs. They have similar effect with the positive nutrient olesoxime in different growth stages of OPCs. When the three components were used in pairs or in combination, these combinations showed a synergistic effect in promotion of OPCs, and the promotion effect was obviously stronger than that of the nutrition treatment alone or even the positive drug olesoxime.

In short, the current study described an *in vitro* model to test the effects of 2'-FL, OPN, DHA and a nutrient blend consisting

TABLE 4 Effects of different nutritional compositions containing 2′-FL, OPN and/or DHA on the number of MBP positive cells.

Groups	Nutrition composition	Density of MBP positive cells (Mean ± SEM)
Control	Vehicle	44.333 ± 4.447
Positive	Olesoxime	82.167 ± 6.226***
1	2-FL(H^a)	79.00 ± 6.846**
2	OPN(H^a)	73.167 ± 6.215*
3	DHA(H^a)	79.833 ± 7.507**
4	2-FL(L^b)+OPN(L^b)	65.833 ± 8.479*
5	2-FL(L^b)+OPN(H^a)	82.333 ± 7.961***
6	2-FL(M^c)+OPN(M^c)	97.667 ± 7.365***▽
7	2-FL(H^a)+ OPN(L^b)	89.50 ± 8.936***
8	2-FL(H^a)+OPN(H^a)	128.50 ± 8.221***^^^▽▽▽###
9	2-FL(LM^d)+OPN(LM^d)	84.667 ± 7.830***
10	2-FL(MH^e) +OPN(MH^e)	122.833 ± 8.518***^^^▽▽▽
11	2-FL(L^b)+OPN(L^b)+DHA(L^b)	72.667 ± 8.135*
12	2-FL(L^b)+OPN(M^c)+DHA(M^c)	102.00 ± 9.331***^▽#
13	2-FL(H^a)+OPN(H^a)+DHA(H^a)	132.833 ± 10.663***^^^▽▽▽###
14	2-FL(LM^d)+OPN(LM^d)+DHA(LM^d)	97.333 ± 5.818***▽
15	2-FL(MH^e)+OPN(MH^e)+DHA(MH^e)	135.00 ± 9.913***^^^▽▽▽###

*p < 0.05 compared with blank control group, **p < 0.01 compared with blank control group, ***p < 0.001 compared with blank control group.
^Compared with 2′-FL high-dose group p < 0.05, ^^^ compared with 2′-FL high-dose group p < 0.001.
▽Compared with OPN high-dose group p < 0.05, ▽▽▽Compared with OPN high-dose group p < 0.001.
Compared with DHA high-dose group p < 0.05, ### Compared with DHA high-dose group pA< 0.001.
a, high dose; b, low dose; c, medium dose; d, low to medium dose; e, medium to high dose.

of the three nutrients on increasing OPC maturation as well as OL myelination. This most promoting effect was driven by a combined net of nutrient blend, and individual nutrients did not exhibit the same positive effect on myelination, which showing the important of nutrients composition in infant formula. For excise mechanism, the results would need to be further verified with an *in vivo* experiment (35). Besides, studies of gene or protein expressions in OLs in response to the various individual nutrients as well as the nutrient blend are required to identify the signal pathways involved in the myelination related effects in the future (36).

Conclusions

As the most abundant constituents in human breast milk or infant formula, 2′-FL OPN and DHA have numerous beneficial health effects, especially during the intellectual development of infant. Our work elucidates a pro-myelinative effect of 2′-FL, OPN and DHA with neural primary cell *in vitro*. The potential mechanism of the nutrients included induction of oligodendrocyte precursor proliferation, differentiation, maturation and myelination *via* which this effect might be mediated. The study also showed that when the three nutrient components were used in in pair or in combination, their effect on promoting OPCs myelination was significantly better than that of the individual components. The results of this experiment clarified the mechanism of 2′-FL OPN and DHA to promote cognitive development, and provided a solid experimental basis for further optimization of infant formula.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by the Laboratory Animal Center of Peking University Health Science Center.

Author contributions

QX, YZ, MG, and QZ designed the study and manuscript writing. QX, JZ, and DC did the laboratory work in the expression and statistical analysis. JZ, DC, and QZ contributed to data analysis, interpretation, and the revision of articles critically for important intellectual content. All the authors read and approved the manuscript.

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Conflict of interest

Authors QX and DC were employed by the company Heilongjiang Feihe Dairy Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Protein restriction during lactation causes transgenerational metabolic dysfunction in adult rat offspring

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Introduction: Protein restriction during lactation can induce metabolic dysfunctions and has a huge impact on the offspring's phenotype later in its life. We tested whether the effects of a maternal low-protein diet (LP) in rats can be transmitted to the F2 generation and increase their vulnerability to dietary insults in adulthood.

Methods: Female Wistar rats (F0) were fed either a low-protein diet (LP; 4% protein) during the first 2 weeks of lactation or a normal-protein diet (NP; 23% protein). The female offspring (F1 generation) were maintained on a standard diet throughout the experiment. Once adulthood was reached, female F1 offspring from both groups (i.e., NP-F1 and LP-F1) were bred to proven males, outside the experiment, to produce the F2 generation. Male F2 offspring from both groups (NP-F2 and LP-F2 groups) received a standard diet until 60 days old, at which point they received either a normal fat (NF; 4.5% fat) or a high fat diet (HF; 35% fat) for 30 days.

Results: At 90 days old, LPNF-F2 offspring had increased lipogenesis and fasting insulinemia compared to NPNF-F2, without alteration in insulin sensitivity. HF diet caused increased gluconeogenesis and displayed glucose intolerance in LPHF-F2 offspring compared to LPNF-F2 offspring. Additionally, the HF diet led to damage to lipid metabolism (such as steatosis grade 3), higher body weight, fat pad stores, and hepatic lipid content.

Discussion: We concluded that an F0 maternal protein restricted diet during lactation can induce a transgenerational effect on glucose and liver metabolism in the F2 generation, making the offspring's liver more vulnerable to nutritional injury later in life.

KEYWORDS

metabolic programming, maternal malnutrition, steatosis, thrifty phenotype hypothesis, metabolism

1. Introduction

A relationship between malnutrition and chronic diseases has been observed worldwide (1). Exponential evidence indicates that perinatal environmental factors, such as maternal malnutrition status, promote long-term effects on the metabolic phenotype of offspring (2, 3); this process is known as metabolic programming. Epidemiological and experimental studies suggest that early life nutritional programming is associated with a higher risk for the development of cardiometabolic syndrome (4–6); an impaired capacity to maintain energy balance is not only limited to exposed individuals but also subsequent generations, even though nutritional conditions are favorable (7).

According to the thrifty phenotype hypothesis, maternal malnutrition provokes metabolic adaptations in offspring that support further development and survival by altered intrauterine growth and an adjusted metabolic phenotype, with a reduced energy demand appropriate for poor nutritional conditions (8–11). However, even an adequate, or excessive postnatal food supply in later life may have negative consequences, mainly on glucose homeostasis (12) and hepatic lipid metabolism (13). This notion is supported by studies on perinatal nutrient restriction during critical periods of development.

Several reports have also focused on the effects of maternal malnutrition. For example, a low-protein diet suggests that the programmed metabolic dysregulation observed in different vulnerability windows may be associated with different biological mechanisms and have a great impact on the phenotype induced in the offspring (7, 14).

Lactation is a window of susceptibility due to the development and maturation of major organs and tissues, which determine the offspring's metabolic phenotype (2, 15). Thus, exposure to undernutrition during this period can affect metabolism and pancreatic function. Our research group showed that a low-protein diet during lactation increased glycemia, even though offspring displayed higher peripheral insulin sensitivity and lower fasting insulinemia (15). Insulin is a major anabolic hormone involved in hepatic metabolism and dietary protein malnutrition can induce hepatic fat accumulation (16). Maternal low-protein diet effects have also been observed in several studies (17, 18) in the F2 (19, 20) and F3 generations (21, 22), contributing to the early life origin of the risk of chronic diseases.

While the investigation of the transgenerational effects of developmentally programmed traits is widening, very few studies have explored the potential for these traits to be transmitted with post-weaning diets other than adequate controls. In the present study, we assessed the transgenerational transmission of programmed phenotype outcomes on glucose homeostasis and lipidic hepatic metabolism through the maternal lineage and offspring vulnerability to a food insult later in life with a high-fat diet.

2. Materials and methods

2.1. Ethical approval

All experiments were conducted according to ARRIVE guidelines (23) and Brazilian Association for Animal Experimentation (COBEA) standards. The protocols were approved by the Ethics Committee in Animal Research of the State University of Maringá (n. 5409020520) and performed in the sectional animal facility of the Secretion Cell Biology Laboratory.

2.2. Dams

2.2.1. Experimental design and diets

After 1 week of acclimatization, female and male Wistar rats (70 and 80 days old, respectively) were mated at a ratio of three females to one male. The pregnant females were transferred to individual cages and fed a standard diet. Pregnant F0 females were used to compose the F1 group (**Figure 1**). At birth, the litter was standardized to eight pups per dam, with a 1:1 sex ratio, and F0 dams were fed either a normal-protein diet (NP; 23% protein; Nuvital; Curitiba/PR, Brazil; n = 12) or a low-protein diet (24) (LP; 4% protein; n = 12) during the first 14 days

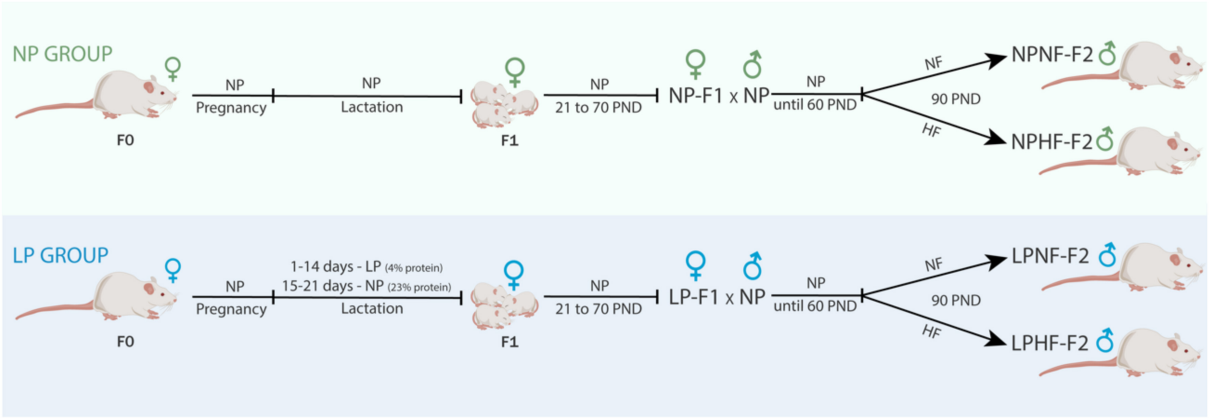

FIGURE 1
Experimental design. NP, normal-protein diet; LP, low-protein diet; NF, normal-fat diet; HF, high-fat diet; PND, post-natal day.

of lactation. At postnatal day 21, the F1 female offspring were weaned, housed in groups of four per cage, and fed a standard diet. The F1 male offspring were not evaluated in the present study.

F1 female offspring (n = 1 per litter) were kept until 70 days old and mated to proven male rats outside the experiment to compose the NP-F2 and LP-F2 groups. F1 females were fed a standard diet throughout the experimental period. After birth, the litter was standardized to eight pups per dam, in a 1:1 sex ratio. After weaning, only male offspring (NP-F2 and LP-F2) were used in the experiments to avoid estrogen influences.

The experimental procedures were conducted at 90 days old. Throughout the experimental period, the animals were kept under controlled temperature (23 C ± 2°C) and photoperiod (7:00 a.m. to 7:00 p.m., daylight cycle) conditions. Animals received water and food *ad libitum*.

2.2.2. Fasting glycemia and oral glucose tolerance test during pregnancy

Glucose concentration was measured via the glucose oxidase method using a commercial kit (GoldAnalisa; Belo Horizonte, MG, Brazil) (25). On the 18th day of pregnancy (26), after a 6 h fast, blood samples were collected before gavage administration of glucose (1 g/kg of body weight, 0 min, n = 6–7) and 15, 30, 45, and 60 min afterward. Glucose response during the test was calculated using the area under the curve (AUC).

2.2.3. Intraperitoneal insulin tolerance test

On the 20th day of pregnancy, an ipITT was performed after a 6 h fast (n = 6–7). Dams received an injection of insulin (1 U/kg of body weight) and blood samples were collected as previously reported (27). Subsequently, the rate of glucose tissue uptake or rate constant for plasma glucose disappearance (K_{itt}) was calculated (28).

2.2.4. Biometric parameters and caloric intake during lactation

Body weight (BW) and food intake were measured daily during the suckling phase. Food intake (FI) was calculated as the difference between the amount of remaining diet (D_f) and the amount presented previously (D_i): [FI (g) = (D_f – D_i)]. Even though the energy values of the diets were the same, food intake was presented in calories (kcal/100 g of body weight). The AUC for food intake and feeding efficiency [food consumption (g)/body weight (g)] was calculated.

2.3. Offspring

2.3.1. Experimental design and diet

At 60 days old, a subset of male offspring from the NP-F2 and LP-F2 groups were fed a normal-fat diet (NF; 7% fat; Nuvital; Curitiba/PR, Brazil) or a high-fat diet (HF; 35% fat) (26) until they were 90 days old. The four experimental groups used were as follows: NPNF-F2, control offspring that were fed an NF diet; LPNF-F2, low-protein offspring that were fed an NF diet; NPHF-F2, control offspring that were fed an HF diet, and LPHF-F2, low-protein offspring that were fed an HF diet (n = 8 litter per group).

2.3.2. Body weight gain, caloric intake, feed efficiency, liver weight, and fat pad store measurements

Body weight (BW) and food intake were determined daily from birth to weaning. They were then examined weekly until they reached 90 days old. Food intake was calculated weekly. Considering that the energetic values of the diets were different, food intake was presented in calories. The AUC for food intake and feeding efficiency were calculated. At 90 days old, the rats were anesthetized with thiopental (45 mg/kg of body weight),

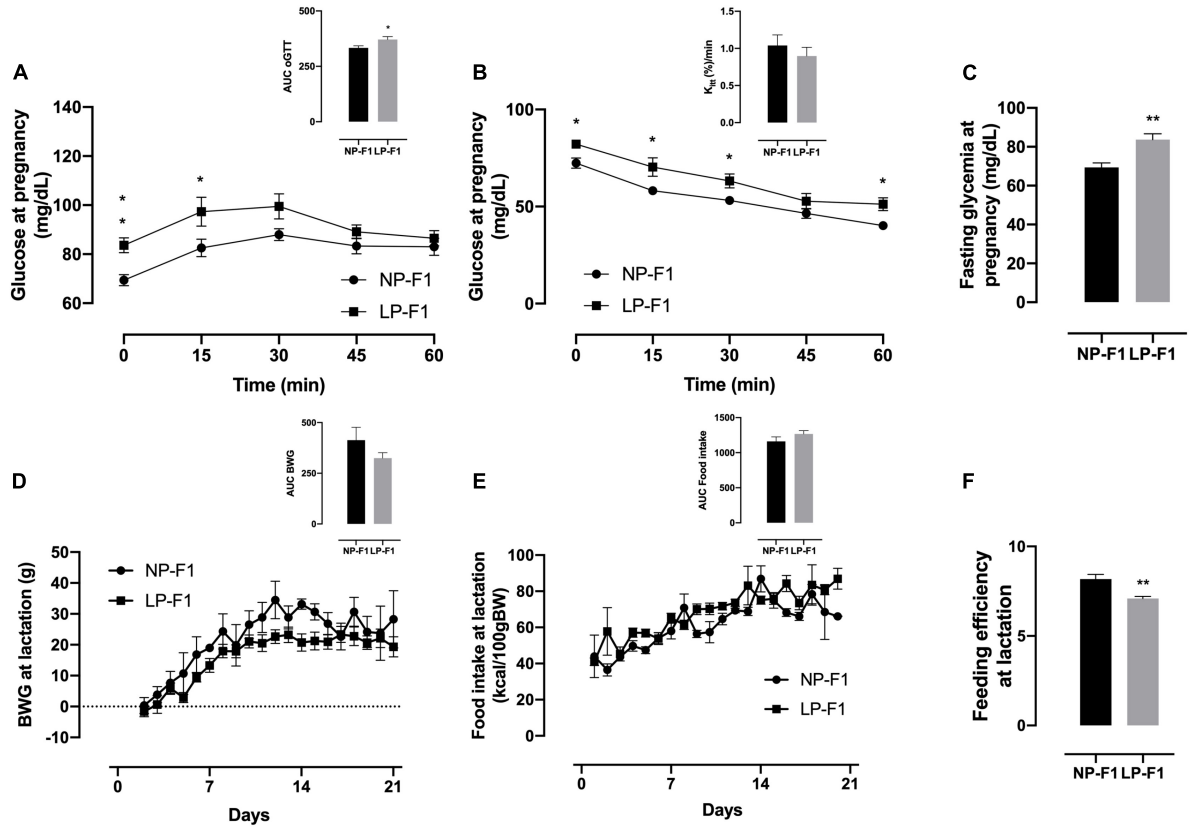

FIGURE 2
Dams' biometric parameters and glucose homeostasis analysis. Plasma glucose during oral glucose tolerance test **(A)**, insulin tolerance test and K_{itt} **(B)**, and fasting glycemia **(C)**, during pregnancy; body weight gain **(D)**, food intake **(E)**, and feeding efficiency **(F)** during lactation. The data are expressed as the means ± S.E.M. and were obtained from 6 to 12 dams (from 6 to 12 different litter). The inset represents the AUC. $*p < 0.05$, $**p < 0.01$ for Student's *t*-test. NP-F1, female rats of dams fed a normal-protein diet during lactation; LP-F1, female rats of dams fed a low-protein diet during lactation.

weighed, decapitated, and laparotomized to remove their liver and retroperitoneal, perigonadal, and mesenteric fat pad stores. The weights of the fat pads and liver were expressed in relation to the BW of each animal (g/100 g of BW).

2.3.3. Intravenous glucose tolerance test

At 90 days old, a batch of animals (n = 10−12 rats from 3 to 4 litters per group) was subjected to a surgical procedure to perform ivGTT, as previously described (24). After a 12 h fast, blood samples were collected before the injection of glucose (1 g/kg of body weight, 0 min) and 5, 15, 30, and 45 min afterward. Glucose response during the test was calculated using the AUC.

2.3.4. Intraperitoneal insulin tolerance test

Another batch of animals (n = 10−12 rats from 3 to 4 litters per group) was cannulated, and ipITT was performed after a 6 h fast. They received an injection of insulin (1 U/kg of body weight), and blood samples were collected, as previously reported (29). Subsequently, the rate of glucose tissue uptake or the rate constant for plasma glucose disappearance (K_{itt}) was calculated (28).

2.3.5. Blood glucose levels and lipid profile

Glucose concentration was measured by the glucose oxidase method using a commercial kit (GoldAnalisa; Belo Horizonte, MG, Brazil) (25). Triglycerides (TG), total cholesterol, and high-density lipoprotein cholesterol (HDL-C) levels were measured in plasma samples using a colorimetric method and commercial kits (GoldAnalisa; Belo Horizonte, MG, Brazil). Low-density lipoprotein cholesterol (LDL-C) and very-low-density LDL cholesterol (VLDL-C) values were calculated using the Friedewald formula (30).

2.3.6. Hepatic levels of cholesterol and triglycerides

Left-lobe hepatic samples of approximately 100 mg were removed (n = 5−10 rats from 5 to 10 litters per group) to determine total lipids using the Folch method (31). The extract was evaporated and then diluted in isopropanol. Cholesterol

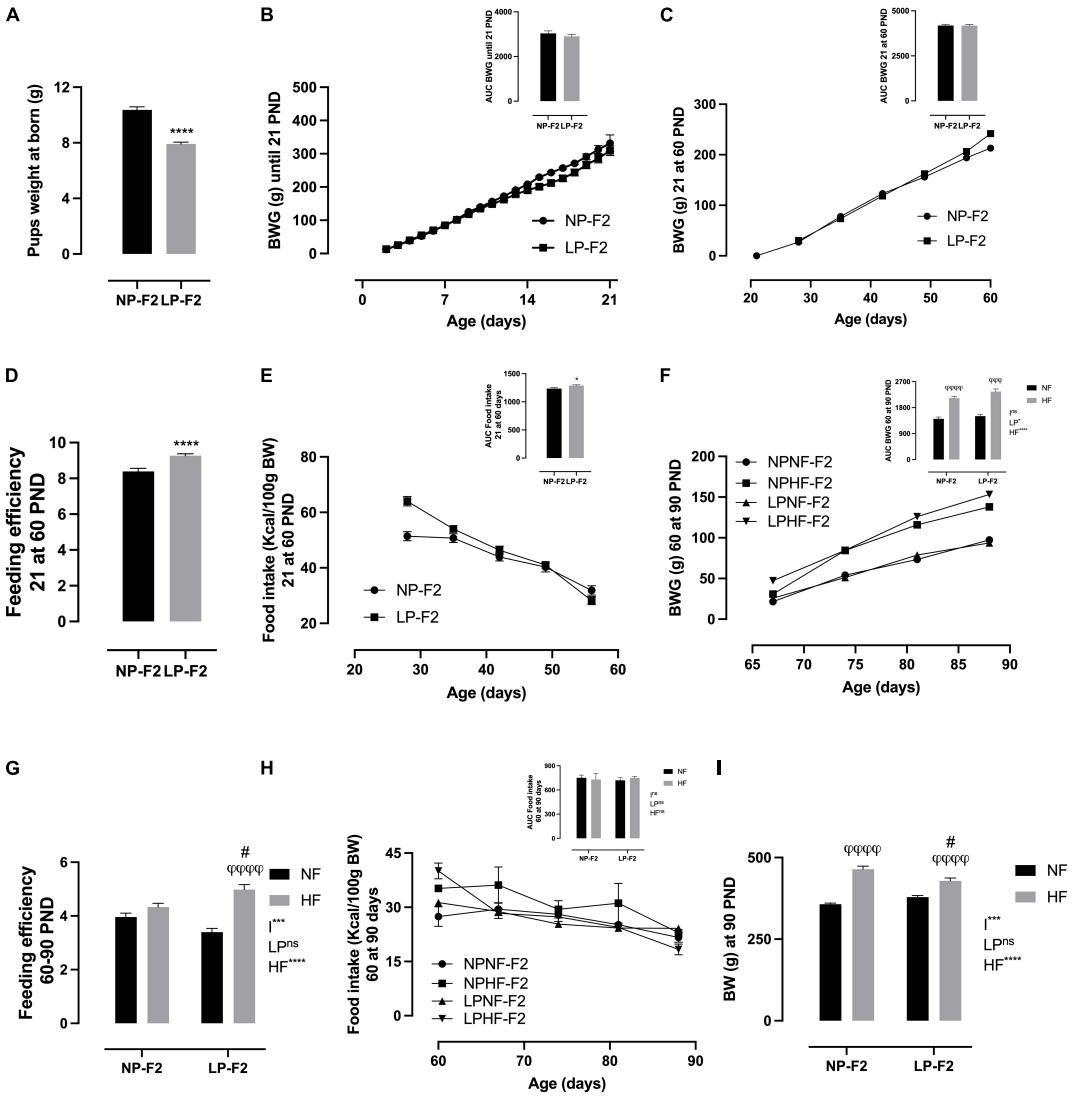

FIGURE 3
Offspring's biometric parameters, caloric intake, and feeding efficiency. Litter weight at born **(A)**, body weight gain during lactation **(B)**, body weight gain from 21 to 60 PND **(C)**, feeding efficiency from 21 to 60 PND **(D)**, food intake from 21 to 60 PND **(E)**, body weight gain from 60 to 90 PND **(F)**, feeding efficiency from 60 to 90 PND **(G)**, food intake from 60 to 90 PND **(H)**, and body weight at 90 PND **(I)**. The data are expressed as the means ± S.E.M. and were obtained from 12 to 15 rats of each group (from 4 to 5 different litters). The inset represents the AUC. $^{*}p < 0.05$, $^{****}p < 0.0001$ for Student's *t*-test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ to NP-F2 vs. LP-F2 in the same conditions; $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, $^{\varphi\varphi\varphi\varphi}p < 0.0001$ to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP, low-protein diet factor; HF, high-fat diet factor; and I, interaction between LP and HF factors. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns, no significant difference, based on two-way analysis of variance.

and TG contents were measured using a commercial kit, in accordance with the manufacturer's instructions (GoldAnalisa; Belo Horizonte, MG, Brazil).

2.3.7. Pancreas, liver, and retroperitoneal fat histology

Pancreas, liver, and retroperitoneal fat samples (n = 5−6 rats from 5 to 6 litters per group) were removed and fixed in 4% paraformaldehyde for 24 h. Subsequently, the samples were dehydrated in an alcohol-increasing series of concentrations. After diaphanization in xylene, the samples were embedded in histological paraffin. Slices of 5-μm thickness were prepared for staining with hematoxylin and eosin (H&E). In the pancreas and fat slices, islets (40 per animal, 40 × magnification) and retroperitoneal fat (20 per animal, 20 × magnification) photomicrographs were randomly acquired using an Olympus DP71 camera coupled to an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan). ImageJ for Windows (Open Source) was used for analysis. Liver slices (30 fields per animal, 20 × magnification) were examined under a

TABLE 1 Effect of high fat (HF) consumption on biochemical parameters at PND90 of adult F2 offspring from F1 dams programmed by protein restriction on lactation.

Parameters	NPNF-F2	NPHF-F2	LPNF-F2	LPHF-F2	LP	HF	I
Fasting glycemia (mg/dl)	94.7 ± 3.0	109.2 ± 5.5$^{\varphi}$	80.2 ± 2.7$^{\#}$	90.8 ± 3.4$^{\#\#}$	ns	****	**
Fasting insulinemia (iU/ml)	153.6 ± 18	160.6 ± 5.5	234.9 ± 7.2$^{\#\#\#}$	253.9 ± 8.8$^{\#\#\#}$	ns	****	ns
Total cholesterol (mg/dl)	87.6 ± 3.8	123.1 ± 4.0$^{\varphi\ \varphi\ \varphi}$	88.7 ± 1.4	101.5 ± 3.9$^{\#\#}$	**	*	****
Triglycerides (mg/dl)	66.1 ± 2.4	82.8 ± 6.4	71.6 ± 4.4	90.7 ± 5.5$^{\varphi}$	ns	ns	**
HDL-C (mg/dl)	51.9 ± 5.1	68.6 ± 2.4$^{\varphi}$	26.7 ± 2.4$^{\#\#\#\#}$	38.9 ± 2.6$^{\#\#\#\#\varphi}$	ns	****	***
LDL-C (mg/dl)	21.2 ± 1.5	30.7 ± 1.6	38.5 ± 1.6$^{\#\#\#}$	53.4 ± 3.1$^{\#\#\#\#\varphi}$	ns	****	****
VLDL-C (mg/dl)	13.9 ± 0.6	18.1 ± 1.2	14.3 ± 0.9	18.1 ± 1.1$^{\varphi}$	ns	ns	***

All data are expressed as the mean ± S.E.M. and were obtained from 10 to 12 rats of each group (from 10 to 12 different litters). NPNF-F2, offspring of the dam (F1) born from NP dam (F0) then received NF during adulthood; NPHF-F2, offspring of the dam (F1) born from NP dam (F0) then received HF during adulthood; LPNF-F2, offspring of the dam (F1) born from LP dam (F0) then received NF during adulthood; LPHF-F2, offspring of the dam (F1) born from LP dam (F0) then received HF during adulthood. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ to NP-F2 vs. LP-F2 in the same conditions; $^{\varphi}p < 0.05$ and $^{\varphi\varphi\varphi}p < 0.001$ to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP, low-protein diet factor; HF, high-fat diet factor; and I, interaction between LP and HF factors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns, no significant difference, based on two-way analysis of variance.

light microscope. These values were classified as previously described for the magnitude of steatosis (32). Thus, steatosis was graded as follows: 0 (none to 5% of hepatocytes affected), 1 (>5%−33% affected), 2 (>33%−66% affected), and 3 (>66% affected). The predominant distribution pattern of steatosis was graded as follows: 0 (zone 3), 1 (zone 1), 2 (azonal), or 3 (panacinar).

2.3.8. RNA isolation and real-time quantitative RT-qPCR

Liver samples were collected and stored in liquid nitrogen at −80°C pending total RNA extraction. RNA was isolated from 100 mg frozen tissue using TrizolTM reagent (Thermo Fisher Scientific, Waltham, MA, United States). The RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm (NanoDrop ND 1000 NanoDrop Technologies, Wilmington, DE, United States). cDNA was synthesized using Platus Transcriber RNaseH cDNA First Strand kit (Sinapse Inc., BR), and quantitation of the tissue expression of selected genes was done by quantitative PCR in the Rotor-Gene® Q (Qiagen) with "HOT FirePol® EvaGreen® qPCR Supermix" (Solis BioDyne, EE). The glyceraldehyde 3 phosphate dehydrogenase (GADPH) gene was utilized as a reference gene. The $2^{-\Delta\ CT}$ method (33) was used for the relative quantification analysis, and data were expressed as an arbitrary unit (AU). Primers for phosphoenolpyruvate carboxykinase (PEPCK) and fatty acid synthase (FASN) are listed in the **Supplementary material**.

2.4. Statistical analysis

The results were normalized and presented as the mean ± standard error (S.E.M.). Statistical analysis was performed using Student's *t*-test or two-way analysis of variance, followed by Tukey's *post hoc* test. $p < 0.05$ was considered statistically significant for the effects of low-protein diet-fed dams (LP), a high-fat diet-fed offspring (HF), or interaction (I) of factors. Analyses were conducted using GraphPad Prism version 6.01 for iOS (GraphPad Software, Inc., San Diego, CA, United States).

3. Results

3.1. Dams

3.1.1. Body weight, food intake, and glucose homeostasis during pregnancy

During the oGTT, as observed by the AUC, LP-F1 dams showed mild glucose intolerance (**Figure 2D**; +10.21%; $p < 0.05$) and fasting hyperglycemia (**Figure 2F**; +17.02%; $p < 0.01$), without differences in insulin sensitivity, as demonstrated by K_{itt} (**Figure 2E**).

As shown in **Figure 2**, protein restriction during lactation did not modify BW gain or food intake in dams (**Figures 2A, B**). However, LP-F1 dams showed a 13.24% decrease in feed efficiency (**Figure 2C**) during lactation ($p < 0.01$).

3.2. Offspring

3.2.1. Biometric parameters and food intake

Protein restriction caused pup weight at birth to be lower by 23.7% in LP-F2 offspring (**Figure 3A**; $P < 0.0001$) compared to the NP-F2 offspring. Metabolic programming showed no change in BW gain between offspring groups during the suckling phase (**Figure 3B**) or until postnatal day 60 (PND) (**Figure 3C**). However, LP-F2 offspring had increased food intake (**Figure 3E**; +24.5%; $p < 0.05$) and feed efficiency

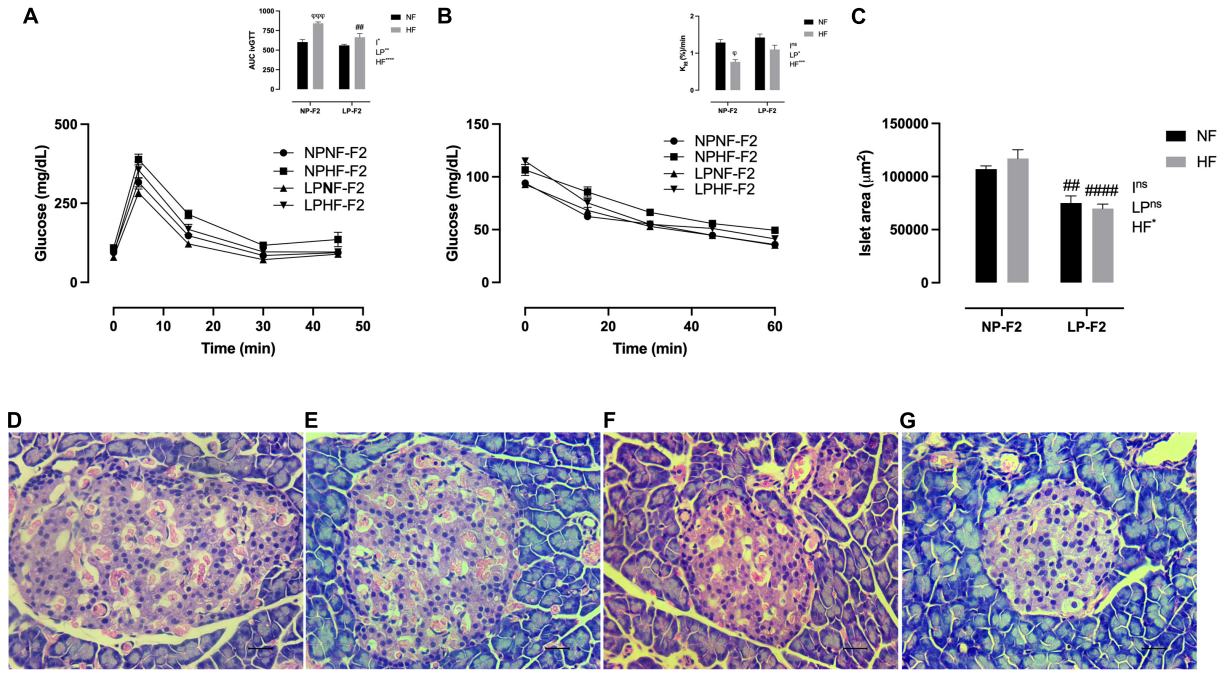

FIGURE 4
Glucose homeostasis and pancreas morphometry at 90 days old. Plasma glucose during intravenous glucose tolerance test (ivGTT) **(A)**, insulin tolerance test and K_{itt} **(B)**, islet area **(C)**, NPNF-F2 islet **(D)**, NPHF-F2 islet **(E)**, LPNF-F2 islet **(F)**, and LPHF-F2 islet **(G)**. The data are expressed as the means ± S.E.M. and were obtained from 6 to 12 rats of each group (from 3 to 6 different litters). $^{\#\#}p < 0.01$ and $^{\#\#\#\#}p < 0.0001$ to NP-F2 vs. LP-F2 in the same conditions; $^{\varphi}p < 0.05$, $^{\varphi\varphi\varphi}p < 0.001$ to NF vs. HF in the same group for the probability based on Tukey's *post hoc* analysis. LP, low-protein diet factor; HF, high-fat diet factor; I, interaction between LP and HF factors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns, no significant difference, based on two-way analysis of variance.

(Figure 3D; +14.54%; $p < 0.0001$) when compared with the control offspring.

Figure 3F shows that there was no difference in BW gain between LPNF-F2 and NPNF-F2 offspring in adulthood. However, exposure to an HF diet resulted in an increase of 33.68% ($p < 0.0001$) and 36.18% ($p < 0.001$) in body weight gain in NPHF-F2 and LPHF-F2 offspring, respectively. No other differences were observed between the NPHF-F2 and LPHF-F2 offspring. Food intake did not differ between groups (Figure 3H). Additionally, feeding efficiency did not differ between the NPNF-F2 and LPNF-F2 offspring. However, the LPHF-F2 group showed an increase of 31.83% ($p < 0.0001$) and 13.07% ($p < 0.05$) in feeding efficiency compared to the LPNF-F2 and NPHF-F2 groups, respectively (Figure 3G), with interactions between factors.

As shown in Figure 3I, at PND90, there was no difference in bin BW between the NPNF-F2 and LPNF-F2 offspring. The NPHF-F2 group had a 23.14% increase in BW compared with the NPNF-F2 group ($p < 0.0001$). Additionally, the LPHF-F2 group has an 11.58% increase in BW compared to the LPNF-F2 group ($p < 0.0001$). Among the groups that received the HF diet, the LPHF-F2 group had a 7.7% decrease in body weight ($P < 0.05$), with interactions between factors.

3.2.2. Biochemical parameters and lipid profile

As shown in Table 1, fasting glycemia was 15.39% lower in LPNF-F2 offspring than in NPNF-F2 offspring ($p < 0.05$). Offering an HF diet, the NPHF-F2 offspring showed a 13.27% increase in fasting glycemia ($p < 0.05$). However, no difference was observed between LPHF-F2 compared with LPNF-F2 offspring. Nevertheless, the LPHF-F2 offspring showed lower fasting glucose (16.89%) than the NPHF-F2 offspring ($p < 0.01$), with interactions between factors.

Fasting insulinemia was 34.6% higher in the LPNF-F2 offspring compared with NPNF-F2 ($p < 0.001$). The NPHF-F2 and LPHF-F2 offspring showed no differences compared to their counterparts. However, LPHF-F2 displayed an increase of 36.75% in fasting insulinemia compared with NPHF-F2 ($p < 0.001$).

Regarding total cholesterol levels, there was no difference between the LPNF-F2 compared with NPNF-F2 offspring. However, as expected, NPHF-F2 offspring had significantly higher total cholesterol levels than NPNF-F2 offspring (+28%; $p < 0.001$). In contrast, HF diet intake showed no statistical difference in total cholesterol between the LPHF-F2 and LPNF-F2 offspring. Interestingly, LPHF-F2 showed lower cholesterol levels than NPHF-F2 (−17.5%; $p < 0.01$), with interactions between factors.

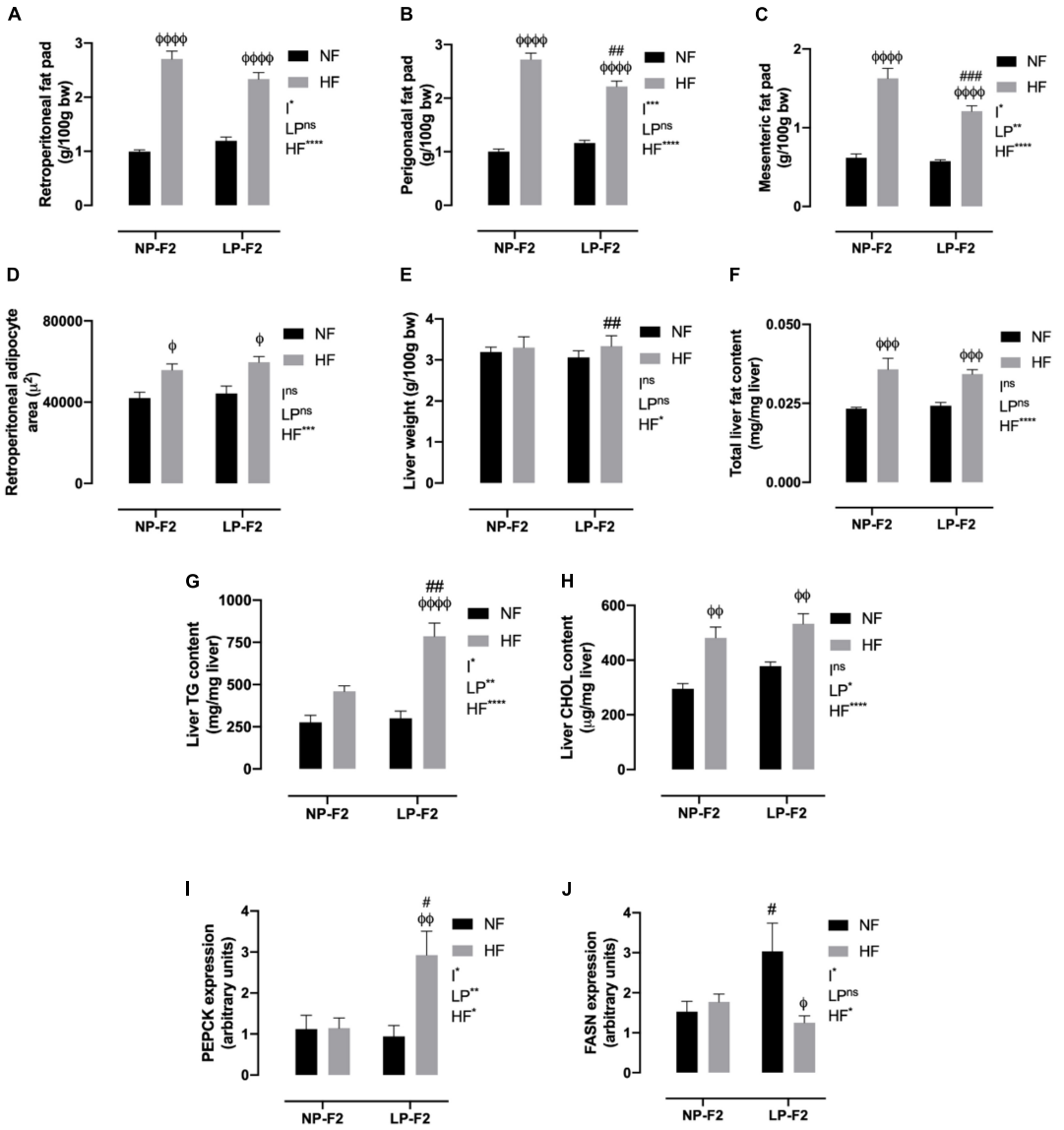

FIGURE 5
Fat pad store and hepatic profile at 90 days old. Retroperitoneal **(A)**, perigonadal **(B)**, mesenteric fat pad store **(C)**, retroperitoneal adipocyte area **(D)**, liver weight **(E)**, total liver fat content **(F)**, liver triglycerides content **(G)**, liver cholesterol content **(H)**, PEPCK **(I)** and FASN expression **(J)**. The data are expressed as the mean ± S.E.M. and were obtained from 8 to 12 rats of each group (from 3 to 4 different litters). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ to NP-F2 vs. LP-F2 in the same conditions; $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, $^{\varphi\varphi\varphi\varphi}p < 0.0001$ to NF vs. HF in the same group for the probability based on the Tukey's *post hoc* analysis. LP, low-protein diet factor; HF, high-fat diet factor; and I, interaction between LP and HF factors. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and ns, no significant difference, based on two-way analysis of variance.

Triglyceride levels did not differ between the LPNF-F2 and NPNF-F2 groups. Additionally, an HF diet did not increase TG levels in NPHF-F2 offspring compared to NPNF-F2 or LPHF-F2 offspring. However, the LPHF-F2 offspring showed an increase of 21.07% in plasma TG levels compared to the LPNF-F2 offspring ($p < 0.05$), with interactions between factors.

The LPNF-F2 group showed a 48.61% decrease in HDL-C levels compared to the NPNF-F2 group ($p < 0.0001$). As expected, HDL-C levels in the LPHF-F2 group were decreased by 43.29% compared to the NPHF-F2 group ($p < 0.0001$). In addition, an HF diet increased HDL-C levels by 24.41% and 31.50% in the NPHF-F2 and LPHF-F2 groups ($p < 0.05$), respectively, compared to their counterparts, with interactions between factors.

In plasma LDL-C levels, the LPNF-F2 group had levels increased by 44.81% compared with the NPNF-F2 group ($p < 0.001$). Moreover, NPHF-F2 offspring showed a tendency to have increased LDL-C levels compared to NPNF-F2 offspring

(p = 0.0944). The LPHF-F2 offspring showed an increase of 27.93% and 42.52% compared with LPNF-F2 ($p < 0.05$) and NPHF-F2 offspring ($p < 0.0001$), respectively, with interactions between factors.

Very-low-density LDL cholesterol levels did not differ between the LPNF-F2 and NPNF-F2 offspring. Curiously, the NPHF-F1 offspring were not significantly different from the NPNF-F2 or LPHF-F2 offspring in terms of VLDL-C levels. However, the LPHF-F2 group showed an increase of 21.07% in VLDL-C levels compared to the LPNF-F2 group ($p < 0.05$), with interactions between factors.

3.2.3. Glucose homeostasis during the glucose and insulin tolerance test

During the ivGTT, as observed by the AUC, the LPNF-F2 group showed no difference in glycemia or peripheral insulin sensitivity compared with the NPNF-F2 group (**Figure 4A**), as demonstrated by K_{itt} (**Figure 4B**). As expected, the HF diet resulted in glucose intolerance (**Figure 4A**; +28.44%; $p < 0.001$) and insulin resistance (**Figure 4B**; −40.48%; $p < 0.05$) in NPHF-F2 offspring compared with NPNF-F2 offspring. However, no differences were observed between the LP-HF-F2 and LP-NF-F2 offspring in glucose levels and kITT. Although the LPHF-F2 offspring showed significantly lower glucose concentrations during the test than the NPHF-F2 offspring (**Figure 4A**; −30.36%; $p < 0.01$), no difference was observed in insulin sensitivity (**Figure 4B**). Glucose tolerance tests showed interactions between the factors.

3.2.4. Pancreatic islet morphometry

Optical analysis showed that pancreatic islet architecture was not altered in the offspring. However, the islet area was lower in the LPNF-F2 (**Figure 4F**) offspring than in the NPNF-F2 offspring (**Figures 4C, D**; −29.75%; $p < 0.01$). The LPHF-F2 (**Figure 4G**) and NPHF-F2 (**Figure 4E**) offspring showed no difference in islet area compared to their counterparts. However, this parameter was decreased by 40.38% in the LPHF-F2 group compared to that in the NPHF-F2 group (**Figure 4C**; $P < 0.0001$).

3.3. Fat pad store composition and morphometry

As shown in **Figures 5A**(C, no difference was observed in fat pad stores between the LPNF-F2 and NPNF-F2 offspring. As expected, the NPHF-F2 offspring had a higher retroperitoneal (**Figure 5A**; +63.30%; $p < 0.001$), perigonadal (**Figure 5B**; +63.19%; $p < 0.0001$), and mesenteric fat pad (**Figure 5C**; +61.98%; $p < 0.0001$) than the NPNF-F2 offspring. Similarly, the white adipose tissue (WAT) mass was higher in the LPHF-F2 offspring than in the LPNF-F2 offspring, with an increase of 48.84% ($p < 0.0001$), 47.63% ($p < 0.0001$), and 52.64% ($p < 0.0001$) for retroperitoneal, perigonadal, and mesenteric fat pad, respectively. While retroperitoneal fat stores were not different, the LPHF-F2 offspring had lower perigonadal (**Figure 5B**; −18.48%; $p < 0.01$) and mesenteric fat stores (**Figure 5C**; 25.68%; $p < 0.001$) compared with the NPHF-F2 offspring, with interactions between factors in all fat pad stores.

Regarding the morphometric analysis of retroperitoneal fat, no differences were observed between the LPNF-F2 and NPNF-F2 offspring. However, the NPHF-F2 and LPHF-F2 groups showed increases of 24.67% and 25.74%, respectively, in the retroperitoneal adipocyte area, compared to their counterparts (**Figure 5D**; $P < 0.05$). No differences were observed between the LPHF-F2 and NPHF-F2 groups.

3.4. Hepatic morphofunction and lipid profile

As shown in **Figure 5E**, the LPNF-F2 group did not show a difference in liver weight compared with the NPNF-F2 group. Thus, an HF diet did not induce any difference in liver weight in the NP-F2 offspring. However, the LPHF-F2 group showed an increase of 8.18% in liver weight compared with the LPNF-F2 group. No difference was observed in liver weight between the LPHF-F2 and NPHF-F2 offspring.

Regarding total liver fat content, no difference was observed between the NPNF-F2 and LPNF-F2 offspring. However, the HF diet increased liver fat content by 36.11% and 29.41% in the NPHF-F2 and LPHF-F2 offspring, respectively, compared with their counterparts (**Figure 5F**; $P < 0.001$). No difference was observed in the LPHF-F2 offspring compared to the NPHF-F2 offspring.

Liver TG content was similar between the LPNF-F2 and NPNF-F2 offspring. Curiously, an HF diet did not induce a difference in liver TG content in the NPHF-F2 offspring compared with the NPNF-F2 offspring (**Figure 5G**). However, liver TG content in the LPHF-F2 offspring increased by 138.17% ($p < 0.0001$) and 158.40% ($p < 0.01$) compared with the LPNF-F2 and NPHF-F2 offspring, respectively, with interactions between factors.

The same pattern of results as for total liver fat content was observed for liver cholesterol content (**Figure 5H**). No statistical difference was observed between the LPNF-F2 and NPNF-F2 offspring or between the LPHF-F2 and NPHF-F2 offspring. However, a HF diet increased liver cholesterol content by 38.72% and 29.08% ($p < 0.01$) in the NPHF-F2 and LPHF-F2 groups, respectively, compared with their counterparts.

The livers of the LPNF-F2 (**Figure 6B**) and NPNF-F2 (**Figure 6A**) offspring exhibited a brown-reddish color with no optical evidence of hepatic lipid alteration. Microscopical

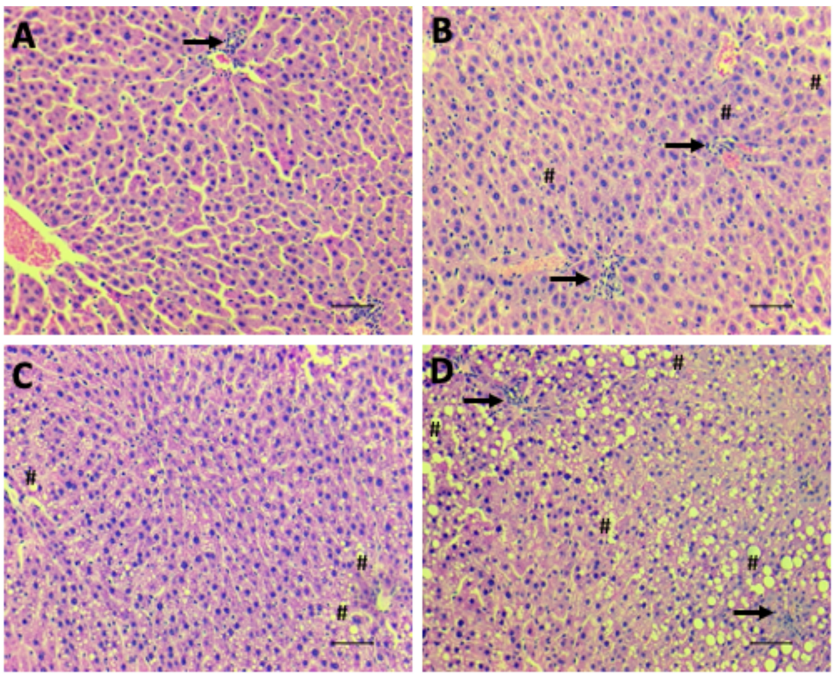

FIGURE 6
Hepatic steatosis. Representative images of microscopical analyses of steatosis grade 0 (none to 5% of hepatocytes affected) (**A**; NPNF-F2), grade 1 (>5%–33% affected) (**B**; LPNF-F2), grade 2 (>33%–66% affected) (**C**; NPHF-F2) and grade 3 (>66% affected) (**D**; LPHF-F2). Hematoxylin and Eosin-stained sections. Hash, examples of macrovesicular steatosis, in all hepatocytes, fat inclusions displaced nucleus to the periphery; Arrow, cluster of inflammatory cells. Magnification: 200×. Scale bar = 50μm.

observation of the hepatocytes showed that they were arranged in rows, delimited by connective tissue containing sinusoids capillaries. As shown in Table 2, NPNF-F2 hepatocytes had a homogeneous cytoplasm without fat vacuoles (score 0; Figure 6A), while the nucleus displayed a central position. LPNF-F2 histopathological analyses showed that 80% of the samples displayed mild steatosis (score 1; Figure 6B) in *zone 1*.

The livers of the HF-fed offspring presented a yellowish aspect, which macroscopically characterizes hepatic steatosis (Table 2). This was confirmed via histopathological analyses, which showed that the hepatocytes of the NPHF-F2 (Figure 6C) and LPHF-F2 (Figure 6D) offspring exhibited displacement of the nucleus to the cell periphery and the presence of large cytosolic fat vacuoles. These analyses demonstrated that 66.67% of the NPHF-F2 offspring displayed severe macrovesicular steatosis (score 3) distributed in zone 1 (50%) or without a distribution pattern (azonal; 50%). In the LPHF-F2 offspring, a HF diet increased severe macrovesicular steatosis (80%; score 3; Figure 6D) evenly distributed throughout the hepatic tissue (panacinar; 60%).

3.5. Liver PEPCK and FASN expression

As shown in Figure 5I, phosphoenolpyruvate carboxykinase (PEPCK) displayed no difference between the LPNF-F2 and NPNF-F2 offspring. Additionally, an HF diet did not induce a difference in PEPCK expression between the NPHF-F2 and NPNF-F2 offspring. However, the LPHF-F2 group showed an increase of 139.10% and 132.17% in PEPCK expression compared with the NPHF-F2 ($p < 0.05$) and LPNF-F2 ($p < 0.01$) groups, respectively, with interactions between factors.

Fatty acid synthase (FASN) was increased by 49.79% in the LPNF-F2 group compared to the NPNF-F2 group ($p < 0.05$) (Figure 5J). Moreover, the NPNF-F2 and NPHF-F2 groups showed no difference in this parameter. FASN expression in the LPHF-F2 group was decreased by 58.75% compared with the LPNF-F2 group ($p < 0.05$). No statistical difference was observed between the LPHF-F2 and NPHF-F2 offspring, with interactions between the factors.

4. Discussion

In this study, we evaluated the transgenerational transmission of the programmed phenotype by LP in the first 14 days of lactation through the maternal lineage to adult male F2 offspring and their susceptibility to damage induced by an HF diet later in life. First, we observed that LP alters glucose homeostasis in LPNF-F2 (second generation) offspring, resulting in lower fasting glycemia and islet area. Insulin

TABLE 2 Effect of HF consumption on hepatic steatosis of adult F2 offspring from dams programmed by protein restriction during lactation.

		% Responses in category				
Item		Score	NPNF-F2 (n = 5)	NPHF-F2 (n = 6)	LPNF-F2 (n = 5)	LPHF-F2 (n = 5)
Steatosis						
	<5%	0	100%	0%	20%	0%
	5%−33%	1	0%	0%	80%	0%
	>33%−66%	2	0%	33,33%	0%	20%
	>66%	3	0%	66,67%	0%	80%
Location						
	Zone 3	0	0%	0%	0%	0%
	Zone 1	1	0%	50%	100%	0%
	Azonal	2	0%	50%	0%	40%
	Panacinar	3	0%	0%	0%	60%

All data were obtained from 5 to 6 rats of each group (from 5 to 6 different litters). NPNF-F2, offspring of the dam (F1) born from NP dam (F0) then received NF during adulthood; NPHF-F2, offspring of the dam (F1) born from NP dam (F0) then received HF during adulthood; LPNF-F2, offspring of the dam (F1) born from LP dam (F0) then received NF during adulthood; LPHF-F2, offspring of the dam (F1) born from LP dam (F0) then received HF during adulthood.

sensitivity was not altered. However, insulin levels were higher. In addition, we showed lipid hepatic alterations with increased lipogenesis and grade 1 steatosis, verifying the transgenerational effects of the grandmother's low-protein diet-fed. After an HF diet was offered during adulthood, we show for the first time that the LPHF-F2 offspring are more susceptible to hepatic damage that the NPHF-F2 offspring. They also showed decreased total cholesterol, HDL-C, and fat pad stores. Furthermore, the hepatic tissue is completely compromised by intracellular fat vesicles. These outcomes corroborate Barker's hypothesis that metabolic programming during critical developmental periods results in altered postnatal metabolism, leaving future generations (such as the F2 generation) more susceptible to diseases (9–11).

The hypothalamus is a well-regulated brain center and an important structure in the control of energy balance (34). The expression of neuropeptides involved in the control of eating behavior is altered in male rats with undernutrition through an increase in the expression of orexigenic hypothalamic peptides, with a concomitant decrease in anorexigenic peptides (35). This results in an increase in caloric intake and a decrease in fat pad stores in adult life (15). In female adult rats, exposure to a low-protein diet during critical periods of development can affect feeding behaviors (36) by causing malformation of the hypothalamus, which remains in adult life (37). However, body weight gain and caloric intake were not altered in F1 dams that were fed a low-protein diet during the suckling phase. In the LP, the removed protein is replaced with carbohydrates to maintain the energy content of the diet. Consumption of these diets can reduce food intake by increasing serotonin production (38).

Several studies have shown that the nutritional status of the dams during critical developmental periods is essential for pups' normal growth and development (26, 38, 39). Maternal milk is considered a better feeding source for newborns (40), and malnutrition during the suckling phase can negatively affect offspring growth, metabolism, and organ development (15, 26). In addition, offspring health is directly influenced by the intrauterine milieu (41). Here, we show for the first time that LP female offspring F1 displayed fasting hyperglycemia and glucose intolerance during pregnancy. Pregnancy is considered a diabetogenic situation *per se*. Maternal hyperglycemia and diabetes can compromise the food supply and induce adaptations in pancreatic fetal development due to glucose transportation through the placenta (41). Pregnant dams with gestational diabetes mellitus produce offspring with normal or low birth weights (39), which show impaired glucose tolerance during adulthood. This diabetogenic effect can be transmitted to the next generation of individuals (41).

Nevertheless, perinatal undernutrition determines a preference for an HF diet and increases dopaminergic action (42), which indicates the vulnerability of pathways that regulate food intake. However, an increase in HF intake was previously not observed in male offspring LP-F1 (15) and LP-F2 programmed during the suckling phase. An HF diet increased feeding efficiency in the LPHF-F2 offspring compared to the NPHF-F2 offspring, demonstrating catch-up growth. This could indicate a higher risk of obesity and related disorders (43).

An HF diet is directly associated with obesity, dyslipidemia, insulin resistance, and glucose intolerance (15, 38). Indeed, our research group previously showed that these parameters were higher in NPHF-F1 adult rats than in LPHF-F1 rats (15). Here, we show for the first time that LPHF-F2 offspring displayed similar results compared to LPHF-F1 male offspring; thus showing non-genomic phenotype transmission by epigenetic mechanisms from the maternal lineage.

Maternal glucose intolerance can harm lipid metabolism and promote fat accumulation in offspring due to the upregulation of *Insr, Lpl, Pparg*, and *Adipoq* mRNA. Adipocyte

hypertrophy is associated with an increase in IL-6 levels, which disrupts insulin signaling (39). The LPHF-F2 group showed high-fat pad gain and an altered lipid profile with a smaller magnitude than the NPHF-F2 group. This transgenerational transmission can contribute to the worldwide pandemic of obesity and type 2 diabetes.

Alterations in lipid profiles have been observed in children with Kwashiorkor syndrome, a deficit in calories and protein. Patients with Kwashiorkor show lower serum TG, and TG accumulation on hepatocytes, due to decreased VLDL-C synthesis. Lower VLDL-C secretion occurs because of a severe protein deficiency. A block in the release of hepatic triglycerides is the major mechanism of fatty liver disease in Kwashiorkor syndrome (44). The LP used in this study had a very low protein content (4%). The restriction of some nutrients in the maternal diet leads to changes in the lipid profile of the offspring (45), such as HDL-C and LDL-C. This is significant as increased LDL-C levels are an important hallmark of cardiovascular disease (46).

A high carbohydrate content in the diet can be associated with decreased HDL-C levels (47). Similarly, LPNF-F2 and LPHF-F2 adult offspring also had lower HDL-C levels, although LP-F1 dams were fed a normal protein diet throughout the experimental period. This profile modification may be due to changes in the expression of the transcription factors that regulate lipolysis and lipogenesis. Suppression of these transcription factors has been shown in rats fed protein restriction during the perinatal period (48), which presented with increased serum TG levels. Triglycerides are the main storage form of energy in adipocytes and hepatocytes in humans and rats. Their release from their stores must be regulated to avoid their toxic potential (49). The liver is an important site for storing excess free fatty acids and is released to control energy homeostasis. TG release occurs in the VLDL-C form by re-esterification at the endoplasmic reticulum with a requirement for apoB and microsomal triglyceride transfer protein (MTP) in the hepatic acinus pericentral zone. Lower VLDL-C secretion leads to the accumulation of TG in hepatocytes, resulting in hepatic steatosis (48).

Hepatic steatosis is characterized by lipid accumulation in hepatocytes, leading to inflammation and the potential progression to liver failure and cirrhosis (50). Even LPHF-F2 offspring had an increase in VLDL-C compared to LPNF-F2 offspring, which is not enough for TG transportation from the liver to the peripheral tissues. Hormonal and dietary factors also affect VLDL-C levels (51). For the first time, we show that protein restriction during the suckling phase had a harmful transgenerational effect on liver tissue when an HF diet was offered. In this study, the LPHF-F2 group displayed higher hepatic TG levels, liver weight, and steatosis grade (score 3) than the NPHF-F2 group.

Fatty acid synthase is one of the major genes responsible for lipid homeostasis and *de novo* lipogenesis and is controlled by hormones and nutritional status. After a meal, blood glucose and insulin levels increase and stimulate *de novo* lipogenesis (52). FASN mRNA expression was higher in the LPNF-F2 offspring, suggesting a compensatory mechanism for energy homeostasis maintenance with low-grade steatosis (score 1). Similarly, a restricted diet during pregnancy increased hepatic FASN mRNA expression in the F0 generation, with a trend in the F1 generation. The increase in FASN mRNA levels correlated with lower HDL-C content in the female progeny (45). Transgenerational studies have shown that LP can affect the hepatic transcriptional profile of thousands of genes until the F3 generation (53). In addition, restricted calorie intake during the preconception or gestational period impairs lipid metabolism, altering FASN mRNA levels in the adult offspring (54). The increase in hepatic TG concentrations during HF feeding is almost entirely driven by uptake and esterification of plasma FFA, without expressive liver contribution. Diets with long-chain fatty acids significantly inhibit *de novo* lipogenesis (55), which could explain low FASN expression in the LPHF-F2 group.

The liver consists of distinct zones with phenotypic heterogeneity, depending on their acinus or lobular localization. Enzymes involved in fatty acid metabolism can exhibit flexibility according to their physiological needs. TG accumulates in hepatocytes starting in the pericentral areas and advancing to the intermediate and periportal areas (56). For the first time, we demonstrated that LPNF-F2 shows steatosis grade 1, with a zone 1 predominant distribution. However, the HF diet increased steatosis to grade 3, with a full distribution of lipids in the liver tissue (panacinar distribution). These results imply that F0 maternal LP could induce a transgenerational effect on liver metabolism and be susceptible to non-alcoholic fatty liver disease in the F2 generation. Liver lipid metabolism is essential to neutralize the impact of FFA-mediated lipotoxicity in peripheral tissues and pancreatic beta cells (56).

The pancreas is the major organ involved in the maintenance of glucose metabolism. Early LP during pregnancy impairs pancreatic beta cell development due to glucose dependence for beta cell maturation, resulting in altered structure and function (14). At the end of gestation, the fetus can control its own glucose levels owing to the adaptation of insulin production and insulin action (26, 41). Studies have shown that poor carbohydrate supply during lactation can change islet structure (37) and stimulate the involution of the pancreas, which is more pronounced in litters from diabetic dams (41). The offspring of hyperglycemic dams display glucose intolerance in adulthood (26). In adulthood, pancreatic mass is normalized (41). In this study, we demonstrated the transgenerational effect of a low-protein diet on glucose homeostasis. The LPNF-F2 group was associated with hypoglycemia and hyperinsulinemia compared to the

NPNF-F2 group. Additionally, the LPHF-F2 group showed lower glucose intolerance and hyperinsulinemia than the NPHF-F2 group, insulin sensitivity was not altered, and the islet area was lower. These structural alterations may be compensatory mechanisms to maintain glucose homeostasis early in life (57).

The liver is the only organ that produces and exports glucose. Liver glucose production is dependent on the PEPCK enzyme, one of the main regulators of the gluconeogenic pathway. PEPCK is activated only after birth and catalyzes oxaloacetate decarboxylation to produce phosphoenolpyruvate in the presence of GTP, which is essential for the gluconeogenesis pathway (58). Nutritional status is the primary target of PEPCK regulation. Lipid accumulation is highly associated with higher PEPCK transcription in Zucker genetically fatty rats (59). In LPHF-F2, PEPCK expression increased. This induction may contribute to gluconeogenesis, which can be associated with glucose intolerance and hyperinsulinemia observed in the LPHF-F2 offspring.

5. Conclusion

F0 maternal protein-restricted diet during lactation could induce a transgenerational effect on glucose and hepatic metabolism in the F2 generation, making liver offspring more vulnerable to nutritional injury later in life. Some studies show that some results are divergent in the F3 compared to the other generations, due to the direct impact of diet restriction on the development of ovum in F2 females, which did not happen in our study. If dietary restriction changes ovum formation, it could have major translational impacts. However, further studies are required to understand the transgenerational mechanisms of a low-protein diet.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee in Animal Research of the State University of Maringá (no. 5409020520).

Author contributions

RV did the conceptualization, performed the methodology, carried out the formal analysis, investigated the data, and wrote the original draft of the manuscript. IM, CM, RC, CZ, AH, WH, TS, and NL investigated the data. TB and JC carried out the resources and supervised the data. AM and PM carried out the resources, supervised the data, and wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Early development of infant gut microbiota in relation to breastfeeding and human milk oligosaccharides

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Background: Infant gut microbiota composition is influenced by various factors early in life. Here, we investigate associations between infant gut microbiome development, infant age, breastfeeding duration, and human milk oligosaccharides (HMO) composition in breastmilk.

Methods: A total of 94 mother-infant pairs were recruited as part of the Cambridge Baby Growth and Breastfeeding Study (CBGS-BF) (Cambridge, UK). Infant stool samples (n = 337) were collected at 2 week, 6 week, 3 month, and 6 month of age. The 16S rRNA V3-V4 rRNA region was sequenced using MiSeq Illumina to determine microbiota composition and diversity. Mother's hindmilk samples were collected at birth, 2 week, 6 week, 3 month, and 6 month postpartum. Concentrations of five neutral [2′FL, 3′FL, lacto-N-fucopentaose 1 (LNFP1), LNnT, LNT] and two acidic (3′SL, and 6′SL) HMOs were measured in all milk samples using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). We explored the associations between infant gut microbiome parameters and age, duration of exclusive breastfeeding (EBF), and levels of individual HMOs.

Results: *Bifidobacterium* was the most abundant genus in infant stool at all-time points, irrespective of breastfeeding duration, with an overall mean relative abundance of 70%. The relative abundance of *B. bifidum* in stool from infants who were breastfed for longer than 6 months was significantly higher compared to the infant breastfed up to 3 months (p = 0.0285). Alpha-diversity (both Shannon and ASV-level Richness) of infant gut microbiota showed a biphasic change with infant age, decreasing from 2 weeks until 3 months and then increasing until 6 months of age. *Bifidobacterium* relative abundance was associated with higher concentrations of 2′FL and LNFP1 in breastmilk across all time-points (p = 0.049 and 0.017, respectively), with trends toward a higher abundance of *B. longum* species. No significant association with *Bifidobacterium* was found for breastmilk LNnT, 3′SL, and 6′SL levels.

Conclusion: Our study is in line with previous data demonstrating that EBF duration in the first months of life impacts infant gut microbiota composition. The observed links between specific HMOs in breastmilk and bacteria in infant stool provide evidence of how mother's milk affects infant microbiome development.

KEYWORDS

microbiome, human milk oligosaccharides (HMO), bifidobacteria, fucosyllactose, clinical

Key message and impact

Our study demonstrates that exclusive breastfeeding during the first months of life significantly impacts infant gut microbiota composition. In a cohort of healthy mother-infant pairs in the UK we observed a trend of decreased microbial diversity in infant fecal microbiota during the first 3 months of age. It was followed by an increased diversity at 3 months of age, which coincides with prominent changes in human milk oligosaccharides (HMO) concentrations in corresponding breastmilk samples from respective mothers during the first 3 months. In our study, select bifidobacteria levels in infant stool were higher in infants who were exclusively breastfed longer (over 6 months) compared to infants breastfed for a shorter period of time (less than 3 months). These associations between levels of specific HMOs in breastmilk and bacteria in infant stool provide important insights into how mother's milk affects infant microbiome development.

Introduction

Gut microbiota plays a critical role in influencing infant growth, body composition, and later life health *via* modulating gastrointestinal, nervous, and immune systems, as well as energy metabolism and fat deposition (1). Infant gut microbiota develops and evolves in the first 1,000 days of life and would attain adult-like composition by the age of 3 years (2). In addition to *Bifidobacterium* spp., anaerobic bacteria such as *Bacteroides* spp. and *Clostridium* spp. have also been identified as colonizers of infant gut during the first 6 months of life and are known to have various effects on infant development and maturation (3).

Infant feeding has been established as one of the major factors shaping he development of gut microbiota (4). Gut microbiome and fecal metabolites of breastfed infants change during lactation and are influenced by breast milk components (5); breastmilk promotes the selective proliferation of a characteristic microbiota. Bacterial families in exclusively breastfed infants have a higher occupancy of *Bifidobacteriaceae* and a lower presence of *Enterococcaceae* and *Enterobacteriaceae* than those in formula-fed infants (6). However, bacterial genera such as *Bifidobacterium* spp. fluctuate dramatically in exclusively breastfed infants (7, 8), which may be partly explained by mother's variation in breastmilk (BM) composition.

Human milk oligosaccharides (HMOs) support bifidobacteria-predominant gut microbiota in breastfed infants (9). HMOs represent the third most abundant solid human milk component after lipid and lactose, even higher than protein (10). With a concentration of 5–10 g/L, HMOs make BM distinct from the milk of other farm animals, including cows, whose oligosaccharide concentrations are 100–1,000-fold lower (10). HMOs composition in BM depends on several factors, including the maternal secretor (*FUT2*) genotype. Fucosylated lactoses such as 2′-fucosyllactose (2′FL), sialylated lactoses such as 3′-sialyllactose (3′SL) and 6′SL, and oligosaccharides with lacto-N-biose structure such as lacto-N-fucopentaose (LNFP) 1 are some of the most abundant HMOs in BM.

HMOs act as selective prebiotics for the gut microbiota (11), and associations between HMOs and infant gut microbiome development have been demonstrated. *Bifidobacterium longum* subsp. *infantis* (*B. infantis)* is the most well-known species that is particularly well-suited to colonize the infant gut (12), attributed to its unique ability to consume a large variety of HMOs. As evidenced in a clinical study (13), the administration of *B. infantis* may lead to successful colonization in the gut, whereby its highly selective and acidic fermentation of HMOs increases the production of lactate and acetate and subsequently reduces intestinal pH as well as counteracts gut dysbiosis [reviewed previously (14)]. This potentially includes an important role in the development and maturation of the immune system. Although HMOs are also consumed by other bacteria (e.g., *Bacteroidaceae*), in general, *Bifidobacteriaceae* is the only bacterial family that can substantially convert HMOs to acidic end products that affect stool pH (15). Previously, Bai et al. reported changes in HMOs in the milk of Chinese mothers with different secretor statuses during 6 months of lactation. Those researchers observed the correlations between fucosylated HMOs (e.g., 2′FL) and *Bifidobacterium* spp. in infant stool during early lactation (16). The impact of milk composition on infant stool microbiome was also suggested by Pace and colleagues, who focused on HMO analyses in different geographical locations (17). Finally, Borewicz and colleagues investigated the associations between concentrations of selected HMOs and infant stool microbiota in 24 mother-infant pairs at 2, 6, and 12 weeks of age. Microbiota composition was associated with mode of delivery and breastmilk LNFP1, 3′SL, and Lacto-N-hexaose (LNH) at different time points (18). Despite accumulating knowledge on the effect of HMOs on the infant gut microbiome [summarized elegantly in a recent review by Masi and Stewart (19)], clinical and cohort studies confirming associations between HMOs levels and infant gut microbiota composition in infant-mother cohorts are still scarce.

In the present analysis, we focused on investigating the associations between early gut microbiota development and composition with infant age, exclusive breastfeeding (EBF) duration as well as individual HMOs by analyzing breastmilk and stool samples during the first 6 months of lactation from 94 mother-infant pairs of the Cambridge Baby Growth and Breastfeeding Study (CBGS-BF) (Cambridge, UK).

Materials and methods

Study design and participants

This study is part of the CBGS-BF, a UK-based prospective observational infant cohort (20). The CBGS-BF is an extension of the original Cambridge Baby Growth Study (21), aiming to investigate determinants of infant growth and body composition. All infants recruited to this cohort were singletons and vaginally born at term from healthy mothers with normal pre-pregnancy BMI and without any significant comorbidities (n = 94) (20). All infants received EBF for at least 6 weeks. Infant stool samples were collected at 2 week, 6 week, 3 month, and 6 month (**Figure 1**). Mother milk samples were collected at birth, 2 weeks, 6 weeks, 3 month, and 6 month postpartum. The study was approved by the Cambridge Local Research Ethics Committee, and all mothers gave written informed consent.

Feeding modules

For statistical analysis, infants were categorized into three feeding groups based on the duration of EBF:

1. Less than 3 months (< 3 month EBF), these infants received EBF for at least 6 weeks.
2. 3–6 months (3–6 month EBF).
3. More than 6 months (> 6 month EBF).

Human milk oligosaccharides analysis

Levels of selected HMOs in maternal breast milk samples were quantitatively determined using high-performance anion-exchange chromatography as reported by Durham et al. (22). The analyzed HMOs included: 2′FL, 3′FL, LNFP1, LNnT, LNT, 3′SL, and 6′SL. For generalized linear mixed models (gLMM) "low" or "high" HMO levels were defined as lower or higher than median levels at each time point).

Infant gut microbiome analysis

Detailed methodology describing DNA extraction from stool samples is provided in the **Supplementary material**. Briefly, 250 mg of fecal sample in S.T.A. R. Buffer (Roche, Indianapolis, IN, USA) were lysed with glass beads. After centrifugation, the supernatant was kept on ice. Purification of DNA was performed on the automated Maxwell instrument (Promega, Madison, WI, USA) by applying the Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega) following the manufacturer's protocol.

PCR amplification of 16S rRNA gene in infant stool DNA, library preparation and sequencing

After DNA extraction, PCR amplification and DNA sequencing of the V3-V4 region of the bacterial 16S rRNA gene was performed in an Illumina MiSeq instrument (Illumina, San Diego, CA, USA). Detailed methodology describing PCR amplification and sequencing is provided in the **Supplementary material**.

Data analysis

A detailed description of analytical methods is provided in the **Supplementary material**. Shannon index and ASV (amplicon sequence variant)-level richness were calculated using the vegan v2.5-7 R package (23). Faith's phylogenetic diversity was calculated using the picante v1.8.2 R package. Weighted Unifrac distances were calculated using the phyloseq UniFrac function. PERMANOVA analyses were performed on the fecal microbiome weighted Unifrac distance matrixes using the adonis function of the vegan R package with 10,000 permutations to estimate the proportion of beta-diversity explained by time point, feeding module, or their interaction, For exclusively breastfed infants only, the effect of individual HMOs—2′FL, 3′FL, LNT, LNnT, LNFP1, 3′SL, and 6′SL—on microbial composition were assessed using PERMANOVA models of weighted UNIFRAC distances at each time point.

Beta-diversity was visualized using Principal Coordinate Analysis (PCoA) performed with the ape v5.5 R package (24) on weighted Unifrac distances.

Generalized linear mixed models (gLMM) were fitted using glmmTMB v1.7.22. Multiple comparison adjustment was performed using the False Discovery Rate (FDR) controlling procedure of Benjamini-Hochberg to limit the FDR to 5% (25). The DHARMa, performance and parameters R packages were used for model diagnostics. All statistical modeling and visualizations were performed in R v4.1.1 using the tidyverse v1.3.1 package. The metacoder v0.3.5 R package was used to create heat tree visualizations. The effect of the individual infant was modeled as a random effect, and an offset term was added to account for differences in library size (i.e., sequencing depth) between the samples.

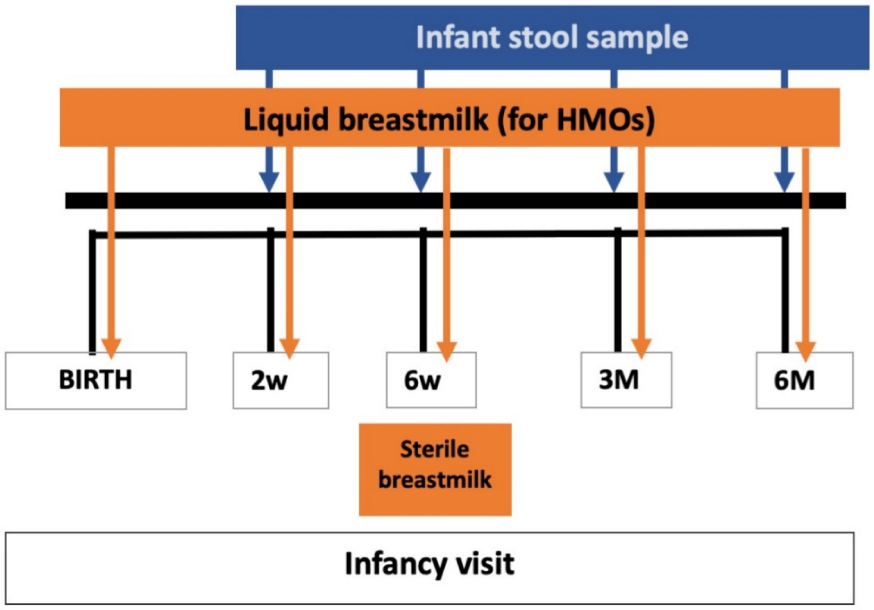
Results

Data was collected from 91 infants and 68 mothers. Infant sex (35 females, 56 males), parity (1 for 35 infants, 2 for 47 infants, 3 for 7 infants, and 4 for 2 infants), and FUT2 secretory status (56 producers, 12 non- or low producers) were included in the analysis as covariates.

Infants included in the analysis were classified based on EBF duration: < 3 months EBF (n = 13), 3–6 months EBF (n = 51), and > 6 months EBF (n = 27; see *Feeding Modules* above).

Infant stool microbiome composition and diversity over time and per feeding module

From 16S rRNA gene sequencing, a total of 1,037 ASVs were detected in the infant stool samples. The fifteen most abundant taxa per feeding module and infant age are shown at genus level (**Supplementary Figure 1A**) and species level (**Supplementary Figure 1B**). Irrespective of breastfeeding duration, the genus *Bifidobacterium* was the most abundant taxon at 2 weeks of infant age (∼50% of infant gut composition) and increased further to ∼75%



Parameters/measurements	Total N of subjects
Total recruited	94
Microbiome analysis	91
HMOs measurement	68
Complete analyses (HMOs and microbiome)	68

FIGURE 1
Timeline for the entire study duration. Fecal and breast milk samples were collected based on specified days (infant's age) as stated for each visit.

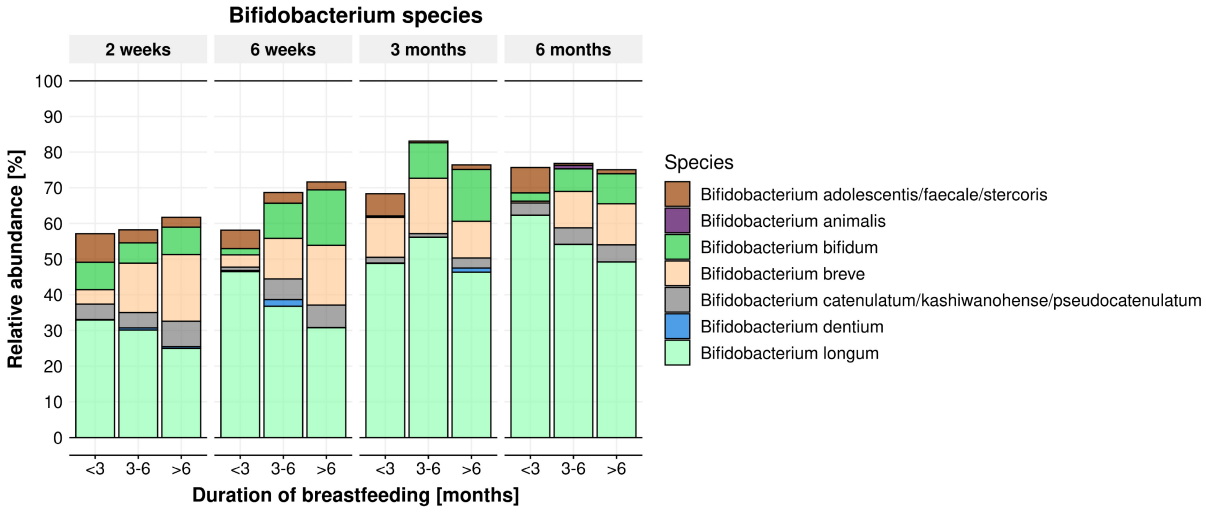


FIGURE 2
Mean relative abundance of *Bifidobacterium* species per feeding module (breastfeeding duration < 3, 3–6, > 6 months) across time (infant age 2 weeks, 6 weeks, 3 months, 6 months). *B. bifidum* abundance was significantly impacted by feeding modules (higher in infants breastfed > 6 month. compared to infants breastfed < 3 month; within the 3–6 months' time frame) ($p = 0.0285$).

of total microbiome composition at 6 months of age (Figure 2; $p < 0.001$). *Bifidobacterium bifidum* abundance was significantly associated with EBF duration (higher in infants EBF > 6 month than EBF < 3 month; $p = 0.0285$). In addition, the abundance of *Enterococcus durans/faecalis/faecium* was lower in both longer EBF groups compared to infants EBF < 3 month (Supplementary Figure 1B). Changes in *Bifidobacterium* relative abundance from 2 weeks to 6 months are shown in Figure 3, with levels increasing from 2 weeks to 3 month for all three EBF groups. Thereafter, the levels of *Bifidobacterium* decreased between 3 and 6 month among infants who remained EBF (EBF 3–6 and > 6 month).

The heat tree shown in Figure 4 provides a visual representation of the global changes in the infant fecal microbiome from age 2 weeks to 6 months. *B. adolescentis/faecale/stercosis* and

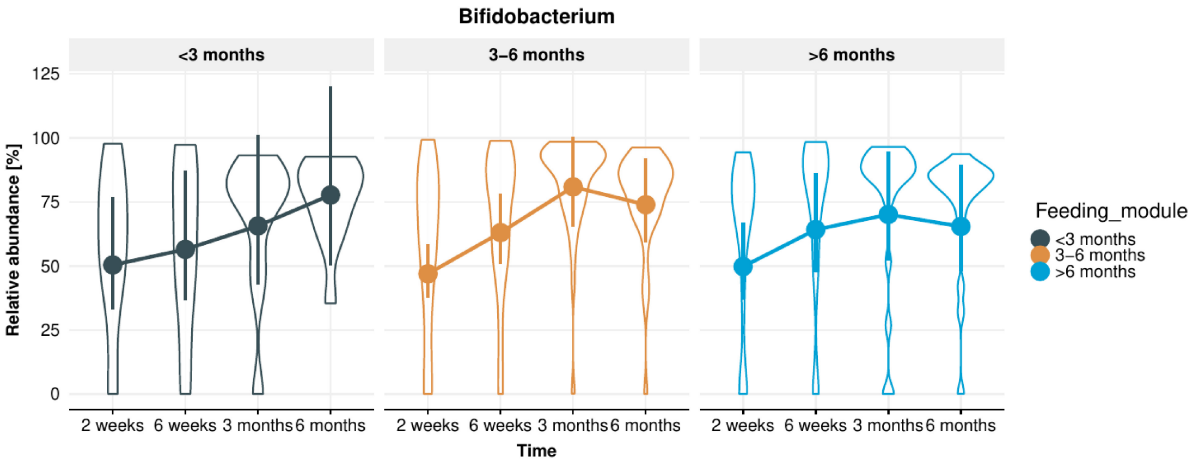


FIGURE 3
Change in *Bifidobacterium* relative abundance in infant stool microbiota from 2 weeks to 6 months of infant age in each of the feeding modules (breastfeeding duration < 3, 3–6, > 6 months). Overall, *Bifidobacterium* relative abundance increased significantly over time ($p < 0.001$).

B. catenulatum/kashiwanohense/pseudocatenulatum were higher at 6 months while other bifidobacteria (e.g., *B. bifidum*, *B. longum,* and *B. breve*) were lower.

The impact of EBF duration on stool microbiome diversity was also evaluated. A longitudinal trend of decreased alpha-diversity (both Shannon and ASV-level Richness) was observed from 2 weeks until 3 months of infant age (except for Richness in infants EBF < 3 month), followed by an increase at 6 months of age (**Figure 5A**). Regardless of EBF duration, Shannon index decreased by 0.13 units from 2 to 6 weeks ($p = 0.0068$), and a further 0.14 units from 6 weeks to 3 months ($p = 0.0022$), but then increased by 0.15 units from 3 to 6 months ($p = 0.00026$). Richness decreased by 2.97 ASVs from 2 to 6 weeks ($p = 0.0022$), was unchanged from 6 weeks to 3 months ($p = 0.50$), then increased by 8.48 ASVs from 3 to 6 months ($p = 2.7 \times 10–8$). Moreover, Richness was significantly lower in longer EBF groups. This difference was significant around 12 ASVs in the 6 weeks to 3 months period ($p = 0.01$) and around 9 ASVs in the 3–6 months period ($p = 0.058$; **Figure 5B**). At 6 weeks, the estimated number of ASVs for infants in the < 3 month. breastfed group was 61 ASVs, while the estimate for the 3–6 months breastfed groups was 49 ASVs (lower for the > 6 month group). At 6 months, the estimated number of ASVs for infants in the < 3 month. breastfed group was around 70 ASVs, while the estimate for the longer breastfed groups was around 61 ASVs.

Beta diversity analysis of the fecal microbiome showed that most of the variance in the weighted Unifrac (quantitative) distances was influenced by inter-individual differences (infants) (R2 = 0.556, $p < 0.001$) and time point (R2 = 0.06, $p < 0.001$). **Figure 6** shows the Principal Component Analysis plot illustrating the intra-individual variance (after removing inter-individual variance) and demonstrates the shift in microbiota composition over time.

Associations between HMOs levels in breastmilk and abundance of *Bifidobacterium* in infant gut

Associations between levels of individual HMOs and *Bifidobacterium* spp. relative abundance in infant gut microbiota were examined using gLMMs. Only samples collected while infants were exclusively breastfed were included in these models ($n = 91$ at 2 weeks, $n = 79$ at 6 weeks, $n = 63$ at 3 months, and $n = 21$ at 6 months). Over time (across all four time points), higher levels of 2′FL and LNFP1 in breastmilk ($p = 0.049$ and 0.017, respectively) were associated with higher *Bifidobacterium* relative abundance in infant stool (**Figure 7**), while a higher LNT level was associated with a lower relative abundance of *Bifidobacterium* ($p = 0.029$, **Supplementary Figure 4A**). LNnT, 3SL, and 6SL levels were not associated with *Bifidobacterium* relative abundance (**Supplementary Figures 4B–D**). Regarding *Bifidobacterium* species, we observed a trend toward higher *B. longum* levels with higher LNFP1 levels ($p = 0.078$). Associations between levels of individual HMOs and relative abundance of other bacterial groups described to metabolize HMOs were either not significant (*Bacteroides;* **Supplementary Figure 5**) or only present in ≤ 2 infant samples and therefore no statistical modeling was possible (*Akkermansia*, *Roseburia,* or *Eubacterium*).

Discussion

Breast milk is ideally the nutritional source for infants since it contains various bioactive components, such as HMOs and antibacterial properties. However, relationships between human milk components, infant gut microbiome, and fecal metabolites throughout lactation remain largely unclear.

This analysis is part of the CBGS-BF recently described by Olga et al. (20). Durham and colleagues reported varying patterns of individual HMO concentrations longitudinally in the same cohort and concluded that the most prominent temporal changes occurred during the first 3 months (22). The present study aimed to analyze infant gut microbiota composition during the first 6 months of life and associations with EBF duration overall, as well as levels of HMOs in breastmilk that could potentially drive changes in microbial communities.

Our study has demonstrated that the duration of EBF significantly affected the infant gut microbiota during the first 6 months of life. We chose to visualize changes in microbiota data for

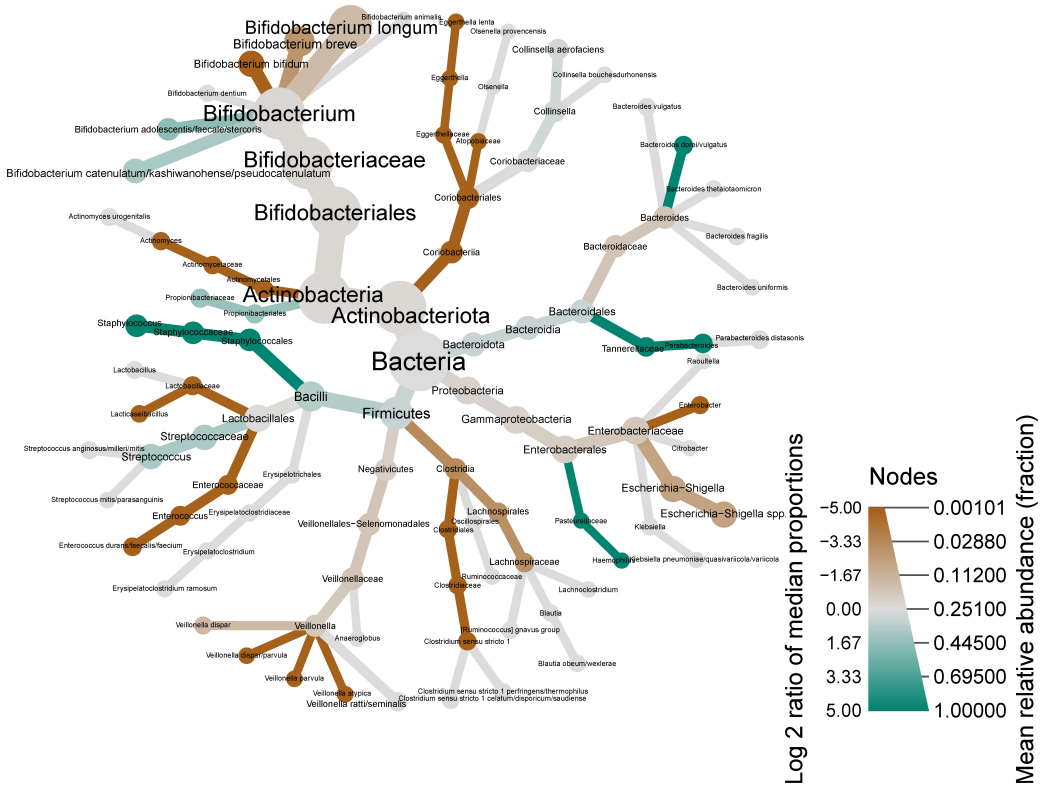


FIGURE 4
Heat tree showing shifts in infant stool microbiome composition between 2 weeks and 6 months of infant age. Nodes represent each taxonomic rank from kingdom (bacteria, center) species (tips of each branch). Node color indicates the log2 of the median proportions of each taxa at the two time points (i.e., green shows an increase, red shows a decrease), while the node size represents the respective taxon's mean relative abundance.

each EBF feeding group separately, even at 2 and 6 weeks (before discontinuation of breastfeeding). This reflects the longitudinal data analysis that was performed. Another reason to separate the EBF feeding groups at 2 and 6 weeks is because infant characteristics (i.e., slower weight gain associated with reduced milk intake were seen in the group who stopped EBF earliest) in the EBF feeding groups already differ before discontinuation of EBF [previously published studies with the same cohort (20, 26)]. There were, however, no significant differences in alpha diversity or Bifidobacterium species between feeding groups at 2 weeks (data not shown)."

It has been reported that the gut microbiome of formula-fed infants is more diverse but less stable compared to breast-fed infants (27). In that study, alpha diversity was lower in breast-fed infants compared to formula-fed infants during the first 3 months after birth but increased significantly at 6 months of age. It has been suggested that higher bacterial diversity in formula-fed infants leads to a shift toward an adult-like microbiome at earlier ages (28). Lower diversity in infants receiving human milk is likely due to the clear dominance of *Bifidobacterium*, which preferentially utilize HMOs as substrates for growth. Komatsu and colleagues indicated that the gut microbiome of breastfed infants fluctuated after month 3 postpartum, even though milk components did not change (5). This likely indicates more efficient metabolism of breast milk components by bifidobacteria. Accordingly, it has been reported that after 3 months of age, the functional maturation of the infant GI tract progresses rapidly, and fecal properties change dramatically (29). In our study, *Bifidobacterium* was the most abundant genus in infant stool at all-time points and irrespective of EBF duration, with an overall mean relative abundance of 70%. Our data shows that levels of *Bifidobacterium* increased until 3 months of age for all infants but decreased during the period from 3 to 6 months of age in infants who were exclusively breastfed beyond 3 months. On the species level, infants EBF for > 6 months showed a higher relative abundance of *B. bifidum* compared to infants EBF for < 3 months. Analyzing the same cohort, Durham and colleagues reported varying longitudinal patterns of individual HMO concentrations and concluded that the most prominent temporal changes occur during the first 3 months (22). In line with the fluctuation of the dominant bifidobacteria, we here describe a biphasic longitudinal trend of initially decreasing alpha-diversity (both Shannon and ASV-level Richness) from 2 weeks until 3 months of infant age followed by an increase until 6 months of age. In addition, richness was lower in infants who were longer EBF.

In our study, levels of selected HMOs in breastmilk were significantly correlated with higher relative abundance of bifidobacteria in infant stool. In particular, levels of 2′FL and LNFP1 were positively correlated with higher bifidobacteria levels, from 2 weeks to 6 months, indicating that bioactive milk components, such as HMOs, contribute to shaping infant gut microbiota composition in exclusively breastfed infants. Another study recently reported positive correlations between HMOs (3′FL and 3′SL) and *B. breve* in infant stool (5). Here, we observed a trend toward higher abundance of *B. longum* in infant stool associated with higher levels of LNFP1 in breastmilk. We could not discriminate between subspecies *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*, but the trend between LNFP1 levels and *B. longum* can

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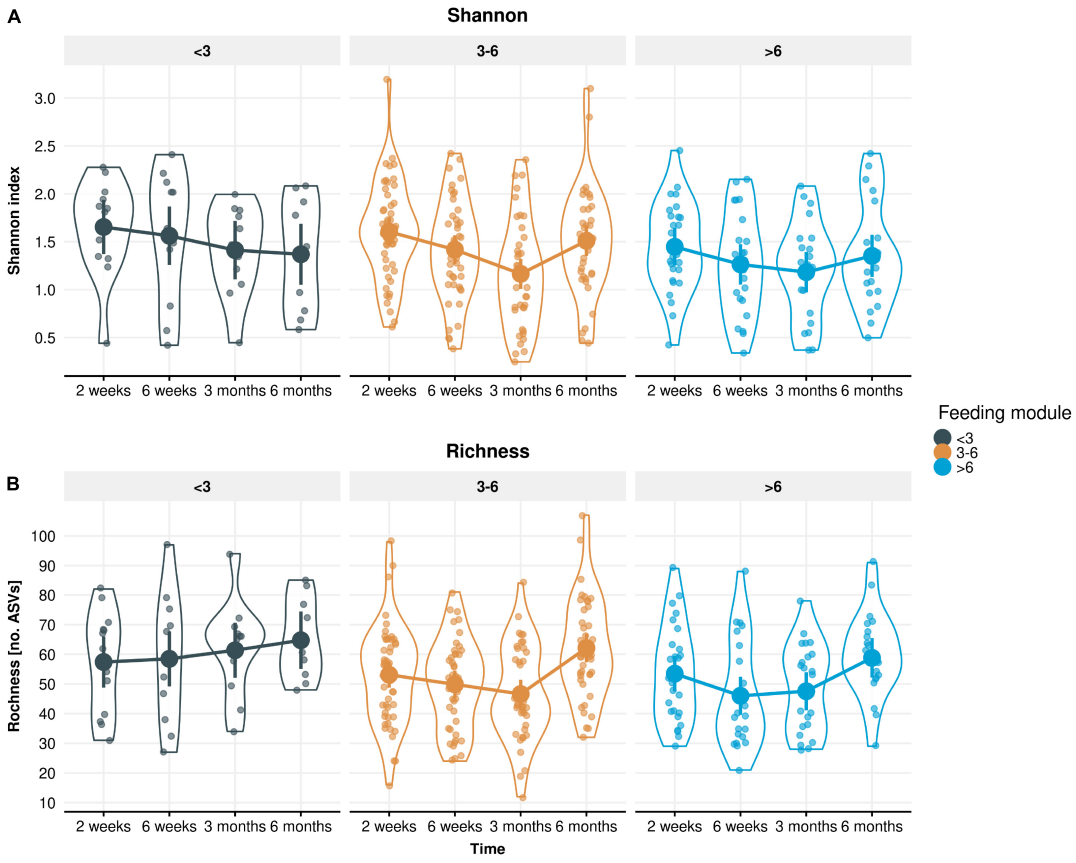

FIGURE 5
Measured alpha-diversity values displayed by **(A)** Shannon index and **(B)** Richness. LMM group estimates with 95% confidence intervals across the four time points (infant age 2 weeks, 6 weeks, 3 months, 6 months) stratified by the three feeding modules (breastfeeding duration < 3, 3–6, > 6 months). Richness was significantly lower in longer breastfed infant groups (p = 0.01 in the 6 weeks to 3 months period and p = 0.058 in the 3–6 months period).

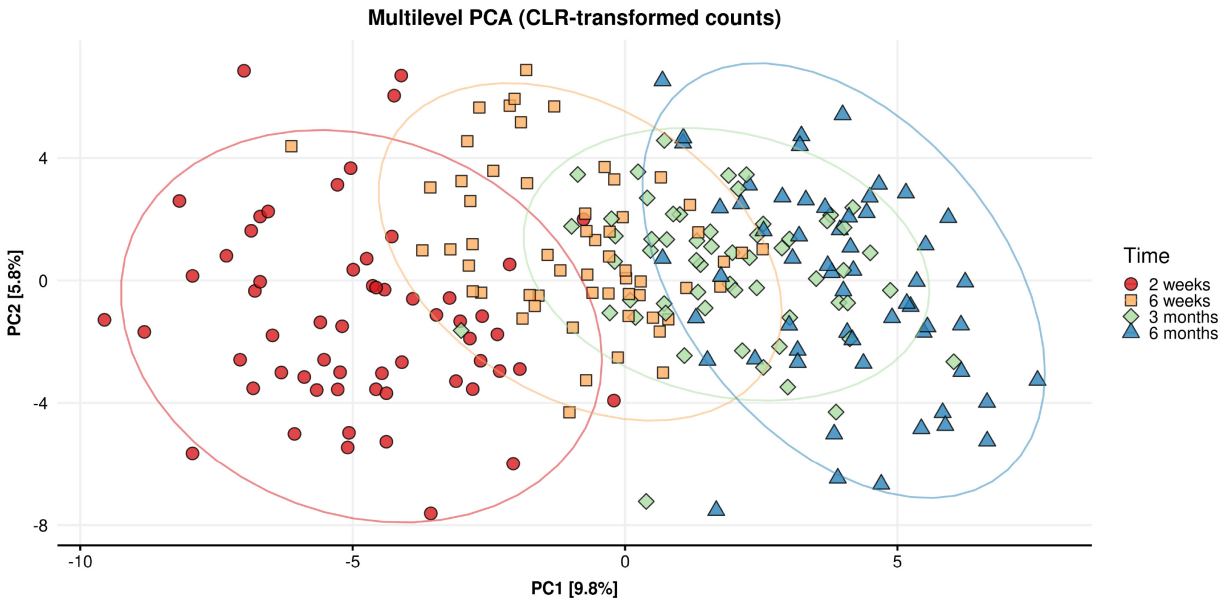

FIGURE 6
Multilevel principal component analysis plot of CLR-transformed microbial counts for infant stool microbiota composition across time (infant age 2 week, 6 weeks, 3 months, 6 months). Only intra-individual variance is decomposed and shown. Inter-individual variance (i.e., between infant differences) is removed.

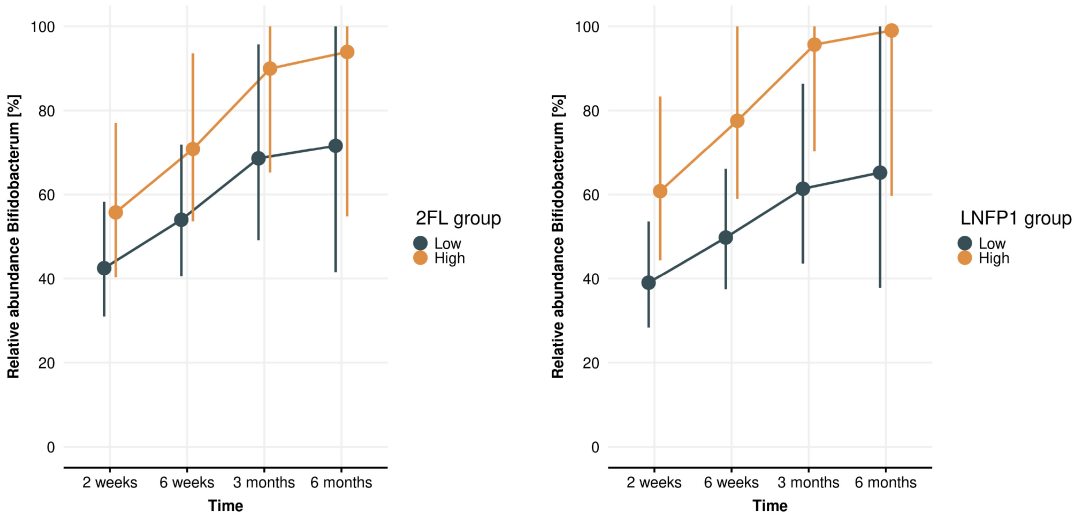

FIGURE 7
Bifidobacterium levels across time (infant age 2 weeks, 6 weeks, 3 months, 6 months) in high and low 2′FL **(left)** and LNFP1 **(right)** groups. Plots show gLMM estimates (95% confidence intervals) of *Bifidobacterium* relative abundance in infants with either low or high HMO levels (where "low" or "high" is defined as lower or higher than median levels at each time point). 2FL (p = 0.049) and LNFP1 (p = 0.017) were significantly associated with a higher *Bifidobacterium* relative abundance.

likely be attributed most to *B. longum* subsp. *infantis*. *B. longum* subsp. *infantis* is equipped with fucosidases that are required to assimilate fucosylated HMOs, while *B. longum* subsp. *longum* generally is not (30). However, no associations were observed between *Bifidobacterium* (spp.) and LNnT, 3′SL, or 6′SL levels. The sialidases, required to utilize 3′SL and 6′SL (31), are also commonly detected in *B. longum* subsp. infantis, but not in *B. longum* subsp. *longum* (30, 32). Nonetheless, specific *Bifidobacterium* species that thrive on specific HMOs would particularly benefit from higher levels of these HMOs in breastmilk, resulting in higher relative abundance. HMO metabolism by *Bifidobacterium* species has been studied mechanistically, in particular using *in vitro* bacterial cultures allowing in depth analyses of specific bacterial strains or consortia and bacterial genes responsible for HMO utilization patterns (33, 34). However, evidence from clinical and cohort studies are limited so far to confirm associations between levels of specific HMOs in breastmilk and abundance of bacterial species in infant gut microbiota. To our knowledge, our present study is one of only few to explore natural variation in HMO levels and its impact on infant gut microbiota composition (35).

Our results provide direct evidence that the infant gut microbiome is dynamically associated with levels of bioactive milk components during a certain lactation period. Since gut microbiota in this sensitive time window in early life plays an important role in the infant's physiology and development of the immune system (36), these insights are important when aiming to support the best start in life and ultimately optimal health outcomes at later ages.

Study limitations

In this study, the microbiome profiling methodology (16S rRNA gene sequencing) did not allow to distinguish subspecies of *B. longum*, therefore we cannot determine the impact of select bioactives on *B. infantis* spp. Next, only a select panel of seven HMOs was analyzed due to lack of reliable standards at time of analysis. On top of the current analysis, it would be of interest to analyze the effects of actual HMO intake instead of breastmilk levels on infant gut microbiota composition. Breastmilk intake volume was only assessed at limited timepoints in the current study. Therefore, analysis on HMO intake would have been underpowered and was not performed.

Conclusion

Our study demonstrates that EBF duration during the first months of life impacts infant gut microbiota composition. *Bifidobacterium* was the most abundant genus in infant stool at all-time points and irrespective of breastfeeding duration, with an overall mean relative abundance of 70%. *B. bifidum* levels were higher in infants who were breastfed longer (over 6 months) compared to infants breastfed for a shorter period of time (less than 3 months). In addition, richness was significantly lower in the longer breastfed infant groups across time points. Links between specific HMOs in breastmilk and bacteria in infant stool as demonstrated in this study provide evidence for how components present in mother's milk affect infant microbiome development.

Data availability statement

The raw sequencing data presented in this study are deposited in the European Nucleotide Archive accession number PRJEB50418.

Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the National Research Ethics Service Cambridgeshire 2 Research Ethics Committee (IRAS No 67546, REC No 11/EE/0068).

Author contributions

GG, JD, MC, LO, KO, and DD conceived and designed the study. AP and GK conducted the microbiome analysis. MC drafted the manuscript. All authors interpreted the data, contributed to manuscripts drafts, reviewed, and revised the manuscript for critical intellectual content, and approved the final version to be published.

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Conflict of interest

The authors declare that this study received funding from Reckitt/Mead Johnson Nutrition. The CBGS BF was funded by a research project award by Reckitt/Mead Johnson Nutrition. The funder advised in the study design, data collection, data discussions and preparation of the manuscript. At the time of study execution MC, JD, and GG were employed by Reckitt/Mead Johnson Nutrition.

AP and GK were employed by the NIZO Food Research BV.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1003032/full#supplementary-material

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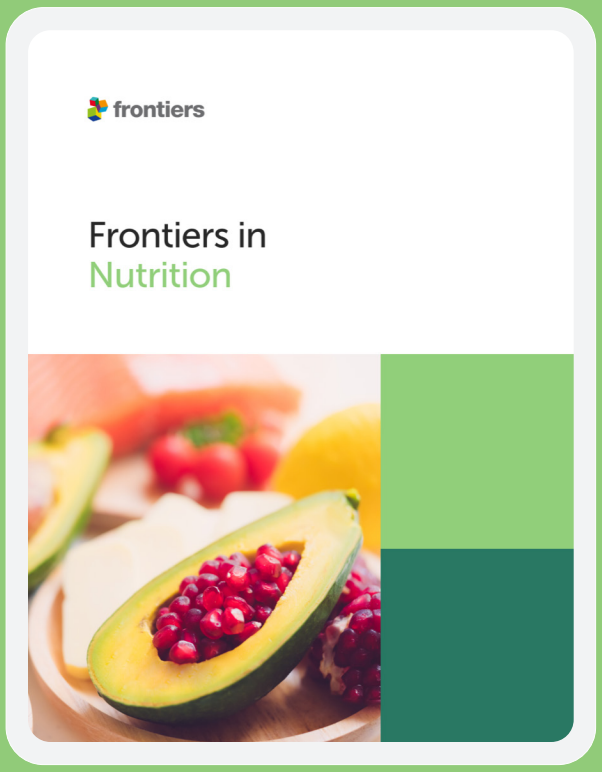

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